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isolation from various kinds of associated aerobic and anaerobic bacteria, and (3) the very great difficulty of isolating certain anaerobic bacteria, notably *B. perfringens*, from mixtures with *B. tertius*.

A critical survey of the literature suggests that *B. tertius* cannot surely be regarded as identical with Rodella's "*Bacillus III*," von Hibler's "*Bazillus IX*," or Fleming's "*Bacillus Y*" as was formerly thought. The *Bacillus gazogenes-parvus* described by Choukevitch in 1911, on the other hand, cannot be differentiated from *B. tertius* by its published description. *B. spermoides* of Ninni (1920) is certainly the same as *B. tertius* according to direct study of Ninni's strain, previously reported by Hall and Matsumura in 1924. But the German identification of *B. tertius* with *B. amylobacter* (van Tieghem), *Clostridium butyricum* (Prazmowski), and Schattenfroth and Grassberger's motile butyric acid bacillus is regarded as untenable.

The present comparative study covered a morphological, cultural, and serological examination of 35 strains of *B. tertius* isolated by the senior author from the following sources:

Human autopsies:	Strains
Peritoneal fluid.....	16
Heart blood.....	4
Uterus.....	1
Pleural fluid.....	1
Lung.....	1
Gall bladder.....	1
Feces:	
Human.....	4
Sheep.....	1
Guinea pig.....	1
Gangrenous wounds:	
Human.....	1
Bovine.....	1
Candy.....	1
Chicken crop.....	1
Home canned corn.....	1

In addition, three strains from other laboratories were examined, including one of the original strains from England, one of "*B. spermoides*" from Italy, and a recent strain of the so-called *B. amylobacter* from Germany.

2. *The Genus Leuconostoc.* G. J. HUCKER AND C. S. PEDERSON, State Agricultural Experiment Station, Geneva, N. Y.

A biochemical study has been made of 80 strains and a survey of 86

additional strains of low acid producing types of streptococci (*Leuconostoc*). These cultures were isolated from slimy sugar solutions, from fermenting vegetables, and from milk and milk products.

A survey of the earlier work pertaining to these organisms indicates that investigators studying fermenting vegetables, including sauerkraut, pickles, tomato products, etc.; those working with milk and milk products, such as starters, organisms from ripening cheese, etc.; and those studying organisms from sugar factories, have been dealing with organisms which are identical or closely related. These organisms belong in one genus.

A study of a series of cultures has shown that they all produce approximately 45 per cent *levo*-lactic acid from glucose, 20 per cent carbon dioxide, and 25 per cent volatile products, including acetic acid and ethyl alcohol. In addition, the members of this group produce mannitol from fructose and sucrose and a levulan or dextran from sucrose.

These fundamental physiological characters give justification for placing these organisms in one genus for which the name *Leuconostoc* has priority. A study of the morphology of the members of the genus *Leuconostoc* has shown that they are intermediate in form between the streptococci and the lactobacilli.

Secondary physiological characters have been used for the recognition of species within the genus. Of these secondary characters, the fermentations of pentoses and sucrose have been found to be the most constant and the species named have been based upon these reactions.

Three species have been recognized in the genus *Leuconostoc*:

1. *Leuconostoc mesenteroides* (Cienkowski) Van Tieghem, ferments pentoses (either arabinose or xylose) and sucrose; produces slime in sucrose solutions; and is generally found in fermenting vegetable material and in sugar solutions.

2. *Leuconostoc dextranicus* (Beijerinck; Hucker and Pederson) which ferments sucrose but not pentoses, produces a moderate amount of slime from sucrose and may be associated either with vegetable or with dairy products.

3. *Leuconostoc citrovorus* (Hammer; Hucker and Pederson) which fails to ferment either of the pentoses or sucrose, produces no slime from sucrose and is generally associated with milk or milk products.

Technical Bulletin No. 167, N. Y. Agricultural Experiment Station, 1930.

3. *Biological and Chemical Studies of the Lactobacillus Genus with Special Reference to Xylose Fermentation.* LOUIS WEINSTEIN AND LEO F. RETTGER, Laboratory of Bacteriology, Yale University, New

Up to within the present decade no work has been done with an organism having as its outstanding characteristic the fermentation of xylose with the production of large amounts of acid. In 1919, Fred, Peterson and Davenport isolated an organism from sauerkraut, corn silage, horse, cow and sheep manures, and from soil, that fermented xylose with the production of lactic acid and comparatively large amounts of acetic acid. They named it *Lactobacillus pentoaceticus*. They found that acetic and lactic acids were produced in a ratio of 42:58, and that the optimum conditions for the production of the acids were incubation at 33°C. and the presence of an excess of calcium carbonate. It was found by them that the lactic acid formed was optically inactive, and that cultivation under anaerobic conditions had no influence on the fermentation process.

The first phase of the present investigation was concerned with a study of the distribution of the pentose-fermenting bacteria in nature. Isolations were obtained from such sources as sauerkraut, corn silage, animal manures, and from soil, using a method involving the preliminary enrichment of the raw material in 0.5 per cent xylose-yeast water, pH 4 to 4.8, and subsequent plating in 0.5 per cent xylose-yeast water agar, pH 7.0. After a few transfers, the organisms were found to produce lactic and acetic acids in large amounts.

The second phase of this work was concerned with a comparative study of the cultural, morphological, and physiological characteristics of *L. pentoaceticus* and various aciduric organisms of intestinal, soil and dental (caries) origin. It was found that the different organisms could not be classified on the basis of morphology, cultural reactions or ordinary physiological properties. A study of the fermentation reactions gave results which did not allow grouping into any separate species. The reactions of a given strain were found to vary from time to time. Too much emphasis should not be placed, therefore, on the sugar reactions as a basis for differentiation between the different members of the lactobacillus genus studied here.

An attempt was made to classify the organisms on the basis of their agglutination reactions, and it was found that the degree of cross-agglutination between the various strains was not sufficiently great to warrant the assumption that the different species were serologically related. Exact correlations between the fermentation reactions and the agglutination reactions could not be established.

The next part of this work was concerned with a study of the acid-producing properties of the pentose-decomposing bacteria. The method

of Friedemann was used for the determination of lactic acid, steam distillation for the estimation of acetic acid, and Youngburg's method for the determination of unfermented xylose. It was found that the optimum incubation temperature for acid production was 33°C. Growth in deep and shallow layer or under anaerobic conditions had no influence on the amount of acid produced. The fermentation was complete in 14 to 16 days, and about 90 to 95 per cent of the pentose was destroyed. From 85 to 92 per cent of the carbohydrate fermented was represented by volatile and non-volatile acids. It was necessary to add calcium carbonate to the fermentation mixture in order to obtain maximum yields. When the cultures were left unneutralized only about one half as much acid was formed as in neutralized mixtures. A comparison of the amounts of acid produced in yeast water and casein-digest media showed that both media were equally well suited for the growth and physiological activities of the pentose-fermenting bacteria.

An experiment was conducted to corroborate Fred and Peterson's observation that lactic and acetic acids are formed in the fermentation of xylose by *Bacterium paratyphosum B*, and to compare the fermentative ability in a roughly quantitative way with that of members of the pentoaceticus group. Six strains of *Bact. paratyphosum B*, five of *Bact. anatum*, two of *Bact. aertrycke* and eleven of a paratyphoid organism isolated by Kulp from squab liver and heart were studied. Three strains of *L. penioaceticus* were carried along in the experiment as controls. The Hopkins-Fletcher reaction and the formation of ethyl acetate showed that both lactic and acetic acids were produced by all the paratyphosum B organisms. The degree to which the pentose was attacked by these was far less than that of the pentoaceticus strains used, the final pH attained by the former organisms never being less than 4.8, whereas with the characteristically pentose-fermenting bacteria it ranged between 3.2 and 3.8. The entire experiment was merely qualitative, because of lack of time, and should, therefore, bear reinvestigation from a strictly quantitative standpoint.

4. *Bacteria Producing Trimethylene Glycol.* C. H. WERKMAN AND G. GILLEN, Department of Bacteriology, Iowa State College, Ames.

Trimethylene glycol is a prominent product of the fermentation of glycerol by certain Voges-Proskauer negative, methyl red positive, citrate and uric acid positive intermediates of the "coli-aerogenes" group of organisms. The yield of trimethylene glycol may reach 30 per cent of the glycerol fermented. Examination of more than 100 cultures of

the "coli-aerogenes" group showed to 11, representing 7 species, which produce trimethylene glycol. Typical *Escherichia coli* and *Aerobacter aerogenes* do not produce trimethylene glycol. Generic recognition is proposed for the group under the genus name *Citrobacter*. The term *Glycolbacter* is inadvisable due to its close similarity to *Glycobacter* previously proposed by Wollman for a genus of amylolytic bacteria.

Generic diagnosis: Gram negative non-sporulating short rods; produce trimethylene glycol from glycerol; citrates serve as the sole source of carbon and urates as the sole source of nitrogen. Fail to produce acetoin from glycerol or dextrose. Methyl red positive (or weakly so). Attack many of the carbohydrates with the production of acid and gas. Nitrates reduced.

Citrobacter freundii isolated and named by Braak (1928) in his monograph on glycerol fermentation is the type species. Descriptions of species will be given in a future publication.

The trimethylene glycol was identified by preparation of the dibenzoate and was separated by fractional distillation from the fermented medium.

5. *The Problem of Ultrafiltration.* C. E. CLIFTON, Department of Bacteriology, Stanford University, California.

This paper discusses: (1) procedures for preparing collodion membranes of graded porosity; (2) results of measurements on membranes prepared by different methods; (3) factors influencing ultrafiltration; and (4) the problem of the filtration of ultrascopic viruses.

6. *The Filtrable Forms of Bacteria.* PHILIP HADLEY, EDNA DELVES AND JOHN KLIMEK, Hygienic Laboratory, University of Michigan, Ann Arbor.

Do filtrable forms of common bacterial species exist? Are these forms "bacterial fragments" or unusually minute examples of the usual morphological type, or are they special cell forms? If they are "special" cells, how do they differ from the commonly known type (S) and how do they arise? Can they be produced at will from the "normal" form, and to what extent are they filtrable?

A laboratory strain of the Shiga bacillus was dissociated under the influence of lithium chloride, of pancreatin, of alkaline broth, and by five other methods. Among the usual dissociates were found, at a certain point in the transformation, minute colonies measuring from 0.2 mm. down to a few micra in diameter. They were at first cultivated with difficulty in any medium and frequently were not visibly cultivable at

all. They were termed the G type colonies and eventually gave rise to the G type cultures.

These cultures (and colonies) were composed of minute granules, globoid bodies, short chains and mycelium. The size of the individual elements varied from one micron down to the limit of vision. They differed from the usual Shiga culture morphologically, culturally, biochemically and serologically; they were non-toxic for rabbits and quite resistant to the influence of Shiga bacteriophage,—both alpha and beta units. Further propagated, they retained their characteristics for numerous generations; but after some weeks returned to the original culture type with all of its attributes. The G type culture is therefore introduced as a distinct cyclostage in the Shiga and other bacterial species, quite as definite and stable as the well known S and R forms with which it is often associated.

Young broth cultures of the G type were invariably filtrable through Berkefeld V, N and W candles, new or old, on 200 mm. of negative pressure and equally well on gravity filtration. Fractional filtration revealed the G forms in every fraction. Inoculation of the fresh filtrates (N or W) into broth or to agar plates gave no immediate sign of growth, and no growth appeared in the incubated filtrates for nine to twenty days or more, after which a slight sediment appeared at the bottom of the tubes. This contained the granular or globoid bodies, which, on further cultivation, yielded the G type culture or colonies. After long continued cultivation, these in turn, "reverted" to the usual Shiga rod type. Cultivation from the fresh filtrates to agar plates usually yielded no visible growth. Further plate-to-plate transfer, however, according to the method of Hauduroy, commonly gave G colonies on the third to eighth plate in the series. These G forms were much more stable than those obtained before filtration.

Fresh filtrates of G type cultures, and young G type cultures themselves, sealed in glass ampoules for more than two years, retained their characteristics, including filtrability. The minute elements of the G type cultures are the most stable form of the Shiga species, rivalling the stability of the S and R forms, which are not filtrable.

We regard these filtrable virus forms as comprising, at least in part, the bacterial microgonidia, liberated from the cells and filaments at a certain point in their ontogeny; and we regard the G type cultures as the first visible cultivable stage lying between the (visible) non-cultivable virus form and the usual cell type. The G type culture and its associated virus-like forms have been produced by us, or by other mem-

bers of our laboratory group, in the following species: *B. coli*, *B. typhosus*, *B. paratyphosus* A, *B. paratyphosus B. enteritidis*, *B. cholerae-suis*, *B. typhi-murium*, *Vib. cholerae*, *B. acidophilus* and *B. diphtheriae* (Park 8). A paper presenting the complete results of this investigation conducted since 1927 is now in press.

7. *Bacteriological Studies Before and After Chlorination at the Madison Sewage Disposal Plant.* BERNHARD DOMOGALLA, City of Madison and Wisconsin State Laboratory of Hygiene.

These studies were undertaken in order to determine what effect pre-chlorination as well as final chlorination has upon the bacteriological flora of domestic sewage as it passes through the new Madison Sewage Disposal Plant,—an Imhoff system. Varying amounts of liquid chlorine were administered to the sewage and its effect upon the bacteria and gas production noted. There was an average drop of about 50 per cent in the total number of bacteria; 90 per cent drop in *B. coli*; 80 per cent drop in hydrogen sulphide formers, and 50 per cent drop in the protein digesters. The rate of nitrification and denitrification showed little change before and after chlorination of the sewage. Due to the great drop in hydrogen sulphide formers after chlorination the odors about the plant were reduced to a minimum. The rate of bacterial gas production in the Imhoff sludge tanks showed no change before and after chlorination.

8. *Dimorphic Sexual Reproduction in Bacteria.* JEAN BROADHURST AND DOROTHY PEASE, Teachers College, Columbia University, New York City.

A lantern demonstration of sexual reproduction in a hitherto undescribed species of *Ascobacillus*, showing conjugation of two dissimilar cells, a minute active rod and a large quiescent spindle- or crescent-shaped cell. These photographs show, also, that the spindle may itself be the result of the previous fusion of two cells, and that the reproductive process may, therefore, include three cells instead of two.

9. *Ergosterol Content of Certain Species of Mycobacterium and the Effect of Ergosterol (Activated and Unactivated) on their Growth.* (Final Report.) PAUL S. PRICKETT AND O. N. MASSENGALE, Research Laboratories, Mead Johnson and Company, Evansville, Indiana.

Following the discovery that the antirachitic vitamin is produced by the activation of ergosterol with ultra-violet light this sterol has assumed much importance and search has been made by many workers for its

sources. The fungi being characterized by a relatively large ergosterol content, many representative species including true yeasts, pseudo yeasts, molds, mushrooms and bacteria have been investigated by us. As the members of the genus *Mycobacterium* contain relatively large amounts of lipoids and because ergosterol is closely allied with the lipid fraction of a material, the ergosterol content of nine cultures, representing the following species, was studied: human and bovine strains of *M. tuberculosis*; *M. avium*; *M. leprae*; *M. smegmatis*; *M. berolinensis*; and *M. phlei*.

Each of these cultures was grown on 5 per cent glycerol-nutrient agar for periods of one week and one month respectively when they were harvested. None of these cultures were found to contain ergosterol when assayed by the spectrographic method. Apparently these are the first fungi in which no ergosterol has been found; the absence of this sterol in these organisms is all the more surprising when their high lipid content is considered.

These same cultures were grown on 5 per cent glycerol-nutrient agar slants to which had been added activated ergosterol (potency 250,000 times that of cod liver oil, according to the Shipley Line Test) and unactivated ergosterol in amounts varying from zero to 6.25 mgm. per slant. It was found, in general, that concentrations up to 1.25 mgm. per slant of both activated and unactivated ergosterol had a growth-stimulating effect during the first two weeks of incubation. The unactivated ergosterol showed the more pronounced stimulating effect. Furthermore the added ergosterol, both activated and unactivated, tended to cause more intensely pigmented growths. However, continued incubation caused the differences to disappear so that at the end of eleven weeks incubation all of the tubes inoculated with the same culture were similar in appearance.

10. Do Broth Culture Filtrates Contain a Bacterial-Growth-Inhibiting Substance? L. A. BARNES, School of Medicine, Western Reserve University, Cleveland, Ohio.

Various authors have reported that broth culture filtrates inhibit bacterial growth. It has been claimed, further, that the effect is due to the presence of active agents ("antivirus" of Besredka). The possibility that the entire phenomenon may be due to simple dilution or exhaustion of nutrient materials led to the experiments to be described. The objects of the investigations were: (1) to control the question of dilution of food substances; (2) to separate the elements in the cells from

those in the medium, and to determine the possible presence of inhibitory substances in each.

Filtrates were prepared from the following materials: (1) pancreatin digested broth; (2) broth cultures; (3) solutions of bacterial cells obtained by (a) autolysis, (b) mechanical shaking, and (c) dissolution in sodium hydroxide. Using these various filtrates as diluents, series of tubes of broth were prepared in which the concentration of the filtrate ranged from 5 to 95 per cent. Growth of organisms in such a series was compared with that in a series of tubes of broth diluted with distilled water or saline in amounts corresponding to concentrations of filtrate. Controls of growth in normal broth and in filtrate or diluent only were included in each series.

Results show that, while there is a gradual diminution of growth as the concentration of filtrate increases, the decrease is no greater, or even less, than that observed when water or saline is used as the diluent.

These results are interpreted as being contradictory to the assumption that broth culture filtrates contain an actively inhibitory substance originating as a result of cell disintegration.

11. *The Fermentation of Polyhydric Alcohols by the Colon-Aerogenes Groups of Bacteria.* CHARLES F. POE AND J. THOMAS FIELD, University of Colorado, Boulder.

A study of the fermentation products by members of the colon-aerogenes groups of bacteria was made in media containing *i*-inositol and *d*-sorbitol. The changes in pH and the amount of gas production were measured at stated times. Inositol gave no acid reaction with the *Escherichia* cultures, but gave a distinct acid reaction with the *Aerobacter* cultures. Sorbitol gave a strong acid reaction with all cultures studied. With inositol, measurable amounts of gas were produced by certain members of the *Aerobacter* group, while no visible gas was produced with the *Escherichia* cultures. Sorbitol gave good gas production with all cultures.

A quantitative analysis was made of the products formed with the organisms which gave acid and gas production. In every case carbon dioxide, hydrogen, acetic acid, formic acid, succinic acid, and lactic acid were produced.

12. *Biological Studies of the Acid-Fast Organism.* S. A. PETROFF AND WM. STEENKEN, JR., Research Laboratory, Trudeau Sanatorium, Trudeau, N. Y.

A study on instability of *M. phlei*, *M. timothy*, *M. tuberculosis-avium*

and *M. tuberculosis-bovium* will be presented. Attempt will be made to demonstrate that the environments play a very important part not only in the development of variants, but also in topographical instability of colonies within the same variant. This variability is brought about by changing the environments which may be the medium, carbon dioxide and oxygen tension. Starting with a non-pathogenic chromogenic acid-fast organism having a definite colony characteristic and cultivating it on a special medium, in time it can be so modified that chromogenicity is practically lost. Guinea pigs inoculated with the organism develop good hypersensitiveness and a fair amount of visceral lesion. The modification is gradual.

19. *Dissociation of Myco. leprae.* GUILFORD B. REED, Queen's University, Kingston, Canada.

The colony form of fifteen strains of *Myco. leprae*, obtained from several collections, was examined. Four of these when first plated on Petroff's gentian violet-egg or certain other media showed uniform S type colonies and continued to produce this form of colony after many generations on well buffered slightly acid fluid media; two other strains when first plated showed only uniform R colony types which have proved to be stable on slightly alkaline fluid media; the remaining nine strains when first plated exhibited varying proportions of R and S colony types.

The S type, from at least certain of these strains, after long cultivation in alkaline fluids has produced a few R type colonies which have exhibited all the characteristics and the stability of the original S. Likewise several of the R types after long cultivation in slightly acid fluids have developed a few S types closely resembling the original S types.

In addition to colony form the R and S types are distinguished from each other, (1) by their acid agglutination: in a series of acid buffered solutions the S begins to agglutinate at about pH 3.0, the R at about pH 4.5; (2) by differences in cataphoretic potential; (3) by the oxygen tension for optimum growth of each type; (4) by the rapid depression of the oxidation-reduction potential with growth of the S type and a much slower and less extensive depression with the R type; (5) by a difference in the lipoid content of the R and S; and (6) by a difference in the antigen content of the R and S as shown by complement fixation.

These criteria are being applied to the differentiation of growth forms of other acid-fast species particularly tubercle bacilli.

14. *Colonial Variation of Escherichia coli on Solid Media with Especial Reference to the Production of Rough Colonies.* CHARLES A. HUNTER, University of South Dakota.

For the past several years work on the dissociation of members of the colon group has been under investigation in this laboratory.

Single cell cultures of both the "S" and "R" types were used in these experiments. The results reported deal only with the variation of colonies upon solid media.

It was found that typical "R" colonies could be obtained by growing "S" cultures upon solid media. About 70 solid media of varying composition were tried out and several were found that would produce good "R" colonies. One of the simplest and best media has the following composition: Bacto beef extract 0.9 per cent, Bacto peptone 1.5 per cent, Bacto agar 4.5 per cent, and Bacto dextrose 1.5 per cent adjusted to pH 6.7 to 6.8. This medium is easily prepared by using Bacto nutrient agar (Standard Methods Formula) in quantities three times as much as generally required. Sixty-nine grams per liter are used instead of the usual 23 grams. One and one-half per cent of dextrose is usually added. Although not necessary, it is desirable. The media are generally made up in 200 to 300 cc. quantities, are sterilized, and plates poured. The surface of the agar is dried and streaked with young broth or agar cultures of the "S" type and incubated at 37°C. for twenty to twenty-four hours. These plates show 100 per cent "R" colonies which can not be distinguished from "R" colonies produced by well stabilized "R" cultures.

By varying the composition of the media other types of colonies can be produced.

15. *The Drying of Bacteria and the Viability of Dry Bacterial Cells.* C. N. STARK AND B. L. HERRINGTON, Cornell University, Ithaca, New York.

It has been postulated that perfectly dry bacteria would live practically indefinitely. This belief is in opposition to the view held by others that in the complete absence of water life is impossible. The facts probably can never be definitely determined. Those who believe that really dry bacteria will live forever can always maintain that death occurred as a result of the method of drying the bacteria, rather than from dryness itself. Similarly, it can be held by others that failure of the bacteria to die was due to incomplete drying. The problem is

further complicated by inability at present to distinguish between free water, bound water, and water of constitution.

A special method for obtaining very dry bacteria has been used with the hope of learning more about the survival of bacteria under very dry conditions. In this process of drying bacteria vacuum, CaCl_2 and P_2O_5 were used. This method gives an exceedingly rapid rate of drying to an extremely low moisture content, without exposing the bacteria to unfavorable temperatures. Apparent dryness is obtained in less than three minutes. All but bare traces of permanent gases are removed in this method of drying bacteria.

The organisms which have been tested, named in the order of their ability to remain alive under very dry conditions, are *Streptococcus paracitrovorus*, *Streptococcus lactis*, *Saccharomyces cerevisiae*, *Staphylococcus aureus*, *Staphylococcus albus*, *Bacterium coli*, and *Lactobacillus acidophilus*. For example, approximately two-thirds of the streptococci originally present grew readily in culture media after 97 days under dry conditions, while only two or three per cent of the original number of organisms in the last three cultures named were able to grow. The composition of the media in which the organisms are grown has been found to be an important factor. Tests showed that exposure of extremely dry bacteria to free oxygen gas caused a pronounced killing of the bacteria.

16. *The Bacteriology of Household Dusts.*¹ MURRAY P. HORWOOD, Massachusetts Institute of Technology, Cambridge, Mass.

1. *Quantitative and qualitative aspects.* The purpose of this investigation was to obtain factual information concerning the numbers and kinds of bacteria found in household dusts, emphasis being placed in the qualitative analysis on the possible presence of certain pathogenic bacteria.

Forty-seven samples of household dust were collected, primarily from Metropolitan Boston. Four of these samples came from New York, Philadelphia and Toledo. Of the 47 samples, 31 were obtained from one and two-family residences and the better grade apartment house, 2 from student dormitories in Cambridge and Philadelphia, 3 from the mezzanine of a popular Boston hotel, 3 from an infirmary and general hospital, 3 from the corridors, laboratories and recitation rooms of an educational

¹ These studies were made for the Air-Way Electric Appliance Corporation of Toledo, Ohio. Those who assisted in the work were M. W. Jennison, M. F. Shaffer, Milton Mezoﬀ, Bernard Canter and R. G. Foster.

institution in Cambridge, 3 from shops and offices and 2 from a nurse's home.

Not more than 40 per cent of the samples were reasonably fresh, i.e., less than three days old. The other samples varied in age from two to six weeks at the time of analysis. The samples were collected as aseptically as possible, and whenever they could not be examined immediately, they were stored in carefully sealed, sterile jars until examined. The accepted bacteriological methods of examination were employed throughout the investigation.

The total count on nutrient agar at 20°C. after five days incubation varied from 20,000 to 32,000,000 bacteria per gram of dust. However, 61.8 per cent of the samples showed a count greater than 1,000,000 and 21 per cent a count greater than 5,000,000. At 37°C., after forty-eight hours, the counts on nutrient agar varied from 26,000 to 45,000,000 bacteria per gram, 58.7 per cent of the samples showing a count above 5,000,000. The average count at 37°C. was 4,200,000 and at 20°C., 3,500,000, the ratio between the two being as 1.2:1.

Total counts and acid formers were also determined on purple lactose agar at 37°C. after forty-eight hours, the ratio between the two being as 6:1.

Total counts and hemolytic organisms were also determined on blood agar at 37°C. after forty-eight hours, the ratio between the two being as 12:1.

Tests for lactose fermenting organisms were made in lactose peptone bile at 37°C. and observed after seventy-two hours incubation. No test was considered positive unless 25 per cent gas was obtained. Only 3 of the 46 samples examined, did not show the presence of lactose fermenting organisms. On the other hand, in 19 samples, or 41.5 per cent of those examined, the number of lactose fermenters was greater than 10,000 per gram.

Forty-seven samples were tested for the presence of spore formers in nutrient broth, after heating the dust suspension at 80°C. for ten minutes. Varying amounts of the heated dust suspension were then inoculated into nutrient broth, and incubated at 20°C. for five days. The cultures were then observed for growth. All the samples showed the presence of spore formers. Of the 47 samples examined, 24, or 51.1 per cent, showed the presence of more than 50,000 spore formers per gram of dust.

Observations for the presence of streptococci were made in two ways: (1) by examining 402 smears made from hemolytic and non-hemolytic

colonies developing on blood agar; and (2) by examining smears made from meat infusion nutrient broth cultures of 44 samples of dust. The reaction of the broth was adjusted to pH 7.2 to 7.4. By this method, streptococci were found in 35 of the 44 samples, or 79.6 per cent. Long chain streptococci, defined as having 10 cells or more, were found in 21 samples, equivalent to 47.8 per cent, and short chain streptococci, defined as having between 6 and 9 cells, were found in 34 samples, equivalent to 77.3 per cent.

Of the 402 colonies from blood agar plates that were smeared and examined microscopically, 255 were hemolytic and 147 were non-hemolytic. The colonies represented 45 samples of household dust. Hemolytic streptococci were isolated in this way in 20 samples, equivalent to 44.5 per cent, and non-hemolytic streptococci were isolated in 14 samples, equivalent to 31 per cent. Hemolytic or non-hemolytic streptococci were found in 60 per cent of the samples examined. Several smears showed diplococci and these should probably have been included with the streptococci, but this was not done. The finding of hemolytic streptococci in dust does not mean that they are necessarily pathogenic, since the pathogenicity of pure cultures of these organisms was not determined by animal inoculation tests.

In the microscopic examination of smears made from the colonies on blood agar plates, hemolytic staphylococci were found in 21 of the 45 samples of household dust examined, equivalent to 46 per cent, and non-hemolytic staphylococci in 27 of the 45 samples, equivalent to 60 per cent. When considered together, staphylococci were found in 33 of the 45 samples, equivalent to 73 per cent.

2. *Effect of moisture and organic matter on bacterial content.* In view of the very high counts obtained in the bacteriological examination of household dusts, and the extraordinary number of vegetative bacteria, some of which undoubtedly have pathogenic significance, it was considered desirable to determine the moisture and organic content of the dusts under consideration. It should be remembered that household dusts obtained in New England during the winter come from homes where the atmosphere is exceedingly dry—usually under 20 per cent relative humidity. Furthermore, the samples varied in age from two to six weeks before being examined. These factors should have made for very low counts and the absence of vegetative bacteria, if the latter are readily destroyed by drying. A total of 41 samples was examined. All the samples showed less than 6 per cent moisture; 29 samples or 70.7

per cent showed less than 4 per cent moisture; and 3 samples showed less than 2 per cent moisture. In spite of such low moistures, the concentration of vegetative bacteria per gram of dust was very high. Of the 41 samples examined for organic matter, all showed the presence of at least 20 per cent organic matter, and 26 samples, equivalent to 63.5 per cent, contained more than 50 per cent organic matter. It seems proper to conclude that in the presence of organic matter in considerable concentration, vegetative bacteria in household dusts survive for long periods of time, in spite of the low concentration of moisture which is present.

3. *Results of animal inoculation tests.* Nine samples of dust were tested for the presence of pathogenic bacteria and particularly for tubercle bacilli. Of the 9 samples, 3 came from private residences, 1 from an apartment house, 1 from a maternity ward of a general hospital, 1 from an office in an educational institution, 2 from men's college dormitories, and 1 from Cambridge street sweepings. Guinea pigs were employed for the tests, the inoculations being made in one set of animals with sterile, saline washings of the dusts, and in a second set of animals, with acidulated sterile, saline washings of the dusts, after the acid had been allowed to act on the bacteria for thirty minutes at 37°C. and then neutralized. Control animals were inoculated with sterile, saline washings of the dusts heated to 80°C. for ten minutes. Each inoculated animal received 1.0 cc. of bacterial suspension, representing the bacteria removed from 0.5 gram of dust. Each animal was kept in a separate cage, under the same atmospheric conditions. The diet in each case was the same. Wherever possible the animals were kept for two months before being chloroformed and autopsied.

Eighteen animals were used to ascertain the presence of tubercle bacilli in the 9 samples of dust examined, when sterile saline washings were employed. When acidulated, sterile washings of the dusts were used, 14 animals were inoculated. A total of 14 animals was also used for controls.

In none of the test animals were there obtained positive findings for tuberculous lesions or tubercle bacilli. However, 8 of the 32 test animals died during the experiments, while none of the 14 controls succumbed. Of the 18 animals inoculated with the sterile, saline washings of the dusts, 7 succumbed to various infections other than tuberculosis. Only 1 of the 14 animals inoculated with the acidulated washings of the dusts, succumbed to infection.

17. *The Characteristics of Certain Microorganisms as Modified by the Reaction of the Medium.* E. W. STEARN AND A. E. STEARN, University of Missouri, Columbia.

In the literature are found only sporadic observations on the effect of the reaction of the medium on rate of growth, chromogenicity, morphology, viscosity, virulence, luminescence, motility, pellicle formation, Gram character, sensitivity to dye, isoelectric range, spore formation, enzyme production, acid agglutination, and the differentiation of S and R forms in the cases of certain bacteria. The present study attempts a systematic exploration of the field to determine the significance and nature of such changes, the pH's at which they occur, how persistent they are, and what correlations there may be among them.

B. cereus, *B. subtilis*, several strains of staphylococci, *B. prodigiosus*, *B. pyocyaneus*, *B. coli*, *B. dysenteriae* and several other organisms were cultured in broth adjusted to various pH values, ranging from 5.3 to 8.0 by means of phosphate mixtures, for extended periods, and as many of the above properties noted as were characteristic of the organism under observation.

The results obtained during a five year period indicate:

1. The behavior is an adaptation to an environment which results in changes in characteristics which are related to specific alterations in the chemical nature of the cell protoplasm. Just what these changes are is as yet obscure.

2. Changes in character may be induced even in the optimum pH ranges of growth, though they are more marked beyond these limits.

3. The changes persist through approximately the same number of subculturings in normal neutral media as was required to produce the variant form from the normal organism.

18. *The Bacterial Culture as an Electrical Half-Cell.* BARNETT COHEN, Department of Physiological Chemistry, Johns Hopkins Medical School, Baltimore, Md.

It is well known that bacterial growth is accompanied by a chemical reduction of the culture medium together with a loss of heat and the liberation of oxidation products such as H_2O and CO_2 , etc. The measurement of the over-all intensity of the reduction can be made potentiometrically; and it can be shown that, when the neutralizing effects of atmospheric oxygen are eliminated, the reduction potential mounts appreciably. After growth at 30° for one to two days in a beef extract-peptone-phosphate broth, pH 7, the difference in reduction potential

(in volts) between a few typical cultures and uninoculated controls was found to be as follows:

<i>Bact. dysenteriae</i> (Flexner).....	0.15 to 0.30
<i>C. diphtheriae</i> (Park No. 8).....	0.25 to 0.30
<i>Bact. coli</i>	0.35 to 0.45
<i>B. subtilis</i>	0.50 to 0.60
<i>Proteus vulgaris</i>	0.50 to 0.90

The significance of these different potentials remains to be determined.

In the case of the anaerobes, strict and facultative, the reduction potential tends to approach that of the hydrogen electrode and may even surpass it. For instance, *Cl. welchii* and *Bact. coli* in sugar medium readily produce a hydrogen overvoltage against a noble metal electrode.

In general, it has been found that the potential of a vigorous culture in ordinary media will mount up to about 0.5 to 1 volt over the control. The bacterial culture during the process of energy conversion is in a sense, therefore, a primary electrical half-cell, and as such should conceivably be able to perform work. Its greatest deficiency lies, however, in the fact that its current output is ordinarily very low (10^{-5} to 10^{-6} amp.) and it is very quickly discharged. To measure this minute current and relate it to the different stages of growth, a microcoulometer has been constructed. The results will be reported at another time.

The low capacity of the bacterial half-cell is ascribable to the fact that it is poorly poised at any moment with respect to electromotively active oxidation-reduction products. This situation can be met only partly by increasing the concentration of the organic ingredients of the medium and prevention of access of air. It can be solved by introducing into the medium inorganic or organic substances of the type of potassium ferricyanide or benzoquinone. These, when partly reduced by the activity of the culture, maintain a readily reversible oxidation-reduction system in the medium, i.e., they poise the medium, and furnish capacity to the bacterial half-cell in proportion to their concentration. By this means, we have been able to build a bacterial battery furnishing current of about 2 ma. at a pressure of 35 volts. The unit cell consists of 10 cc. of culture coupled to a sterile control; and it yields about 0.2 ma. at a pressure of 0.5 volt with only small polarization for at least 5 minutes.

These experiments are designed to illustrate the mutual importance of the intensity and capacity factors of biological oxidation-reductions.

19. *A Method for the Determination of Ethyl and Butyl Alcohols in Fermentation Mixtures.* C. H. WERKMAN AND O. L. OSBURN, Department of Bacteriology, Iowa State College, Ames.

The fermented medium is adjusted to neutrality and distilled until one-half to two-thirds has been received as distillate. Fermentation products containing the carbonyl group which interfere, such as acetone, acetoin or acetaldehyde may be precipitated by 2,4-dinitrophenylhydrazine and the alcohols redistilled from an alkaline solution. The alcohols in the distillate are oxidized by adding to 50 cc. of the distillate in a 200 cc. balloon flask, 10 grams of potassium dichromate and 25 cc. of c.p. 85 per cent phosphoric acid. The flask is connected to an efficient reflux condenser. Bring to a gentle boil in one and one-half minutes and maintain for three minutes. Wash condenser with 5 cc. of water at the top. The volume of liquid in the flask should never exceed 100 cc.

After oxidation is complete, the flask is quickly connected to a Liebig condenser and distilled until the mixture in the flask begins to foam. The heat is then lowered until foaming just continues. The heating is then continued for two or three minutes. If this procedure is carried out carefully all of the volatile acid is carried over but none of the phosphoric. The mixture has no tendency to bump or spatter.

The distillate is made up to 100 cc. and the total acid determined by titrating 25 cc. The result should be expressed in cubic centimeters of 0.1 N acid. Thirty cubic centimeters are then partitioned with iso-propyl ether as described by Werkman.

Under these conditions ethyl alcohol is quantitatively converted into acetic acid, and butyl alcohol into a mixture containing 90 per cent butyric acid and 10 per cent acetic acid. This ratio of butyric to acetic acid was determined by oxidizing 5 butyl alcohol solutions varying in concentration from 50 mgm. of alcohol to 600 mgm. per 50 cc. The percentages of butyric acid of total acid were 90, 90.2, 90, 90.5 and 91.

The calculations are most easily made in terms of cubic centimeters of 0.1 normal solutions.

From the nomogram given by Werkman may be read the per cent of butyric acid. This per cent divided by 0.9 and multiplied by the cubic centimeters of 0.1 N acid in the distillate gives the total cubic centimeters of 0.1 N acid derived from the butyl alcohol and designates the cubic centimeters of 0.1 N butyl alcohol in the alcohol mixture.

The difference between the total acid and that representing butyl alcohol gives the cubic centimeters of 0.1 N acid representing the ethyl alcohol. An example will make the meaning clear.

Assume that the distillate contains exactly 100 cc. of 0.1 N acid. The partition constant is found to be 16.5. Reference to the nomogram shows that the mixture contains 60 per cent butyric and 40 per cent acetic acid. Then $\frac{0.60}{0.9} \times 100 = 66.6$ cc. of 0.1 N acid = 66.6 cc. of 0.1 N butyl alcohol. $100 - 66.6 = 33.4$ cc. of 0.1 N ethyl alcohol.

The method was applied to 15 known alcohol mixtures. The maximum error was 3.5 per cent for ethyl alcohol and 3.0 per cent for butyl alcohol.

20. *A Method for the Differential Staining of Gram-Positive and Gram-Negative Bacteria in Tissue Sections.* J. HOWARD BROWN AND LENA BRENN, Johns Hopkins University Medical School, Baltimore, Md.

The methods which have been described for the differential staining of Gram-positive and Gram-negative bacteria in tissue sections have often been found unsatisfactory in that the Gram-negative bacteria are apt not to be sharply stained or differentiated from certain tissue elements. In our hands the following method has given excellent results:

Paraffin sections are prepared as usual for staining.

1. Stain in freshly filtered alum-hematoxylin (Harris) for two to five minutes.
2. Wash in acid alcohol (3 per cent HCl in 95 per cent alcohol) until light pink.
3. Wash in ammonia water (1 cc. *aqua ammoniae* in 100 cc. water) until blue.
4. Wash in water.
5. In a small vial mix 5 drops of 5 per cent aqueous solution of sodium bicarbonate (containing also 0.5 per cent phenol as a preservative) with about 0.75 cc. of 1 per cent (by weight) aqueous solution of gentian violet. Immediately pour the mixture onto the slide and stain for two minutes.
6. Wash quickly with water.
7. Cover with Lugol's iodine solution for one minute.
8. Wash with water. Blot.
9. Decolorize in 1 part of ether plus 3 parts of acetone, dropping it onto the slide until no more color comes off.
10. Blot.
11. Stain for five minutes with rosanilin hydrochloride (0.005 gm. per 100 cc. water) or basic fuchsin (0.1 cc. saturated alcoholic solution per 100 cc. water).
12. Wash in water. Blot but do not allow the section to dry.
13. Pass through acetone.
14. Decolorize and differentiate by dropping over the section a solution of 0.1 gram picric acid in 100 cc. acetone until the section becomes a yellowish-pink. This is the most critical stage of the process and should be carried out by holding the slide over a white plate or dish. Most of the fuchsin should be decolorized from the tissue but the Gram-negative bacteria should remain red.

15. Pass successively through acetone, equal parts of acetone and xylol, and xylol.

16. After clearing in xylol mount in balsam.

(Beginning with step 5 it is best to work with only one slide at a time.)

Cell nuclei should be stained dark reddish-brown; cytoplasm yellowish; Gram-positive bacteria deep violet or almost black; Gram-negative bacteria bright red. Leucocytes generally stand out plainly with a dusky yellowish cytoplasm. Basophilic granules stain red. Red blood cells may be yellow or red depending upon the degree of decolorization in picric acid. Cartilage stains pink. Striated muscle and fibrin generally stain yellow but may retain more or less of the red stain. The tissue stain has many of the characteristics of Van Gieson's stain.

By this method we have been able to stain successfully sections of tissue showing influenza bacilli, pneumococci and streptococci; pertussis bacilli crowded among the cilia of the epithelial cells of the trachea and bronchi; gonococci, staphylococci and streptococci in heart valve vegetations; meningococci in the meninges; *Corynebacterium pyogenes*, *Actinomyces necrophorus*, *Bacillus anthracis*, *Clostridium welchii* and *Clostridium septique* in animal tissues.

21. *A Direct Method for the Determination of the H-ion Concentration of Cultures.* EMIL WEISS, Department of Bacteriology, Pathology and Preventive Medicine, Loyola University Medical School, Chicago, Ill.

Asparagin broth of the following composition is used: asparagin 10 grams, pepton 1 gram, sodium chloride 5 grams, water 1000 cc.

The tubes, containing 10 cc. of the medium and 0.8 cc. of the aqueous solution of the respective indicator are inoculated and incubated. These culture tubes are, from time to time, placed in the comparison block and compared with the colorimetric standard tubes according to Medalia's method. By not removing anything from the culture tubes and comparing them directly with the standard, a continuous study of the H-ion concentration of a given culture is assured and the procedure greatly simplified as compared with Medalia's original method.

22. *Observed Variations in Bacterial Counts on the Same Plate.* F. L. SCHACHT AND A. H. ROBERTSON, New York State Department of Agriculture and Markets, Albany.

Inspection of milk control laboratories in New York State reveals that

one of the greatest sources of error in the agar plate count occurs in the counting of the colonies on the plates. The records of one laboratory showing the results of duplicate or check counting of the same plate by several workers gave a clue to the magnitude of this error. A preliminary test confirmed the recorded results and raised a number of unanswered questions. Among these were: (1) What is the permissible per cent of variation in duplicate counts of the same plate? (2) What is the effect of the use of a magnifying device? (3) What effect has the use of artificial illumination on the counts? (4) Can the personal factor be measured? Obviously, the need of more extensive tests was indicated. These were undertaken in various parts of New York State.

The following system, or a slight modification of it, was used for securing duplicate counts. At each contest six individuals took part in the counting. Six plates with counts ranging from 50 to 250 were selected. Plates showing pin points and spreaders were eliminated. Six counting devices were then selected. The plates and devices were numbered from 1 to 6 and so arranged that each plate was counted twice by each individual, using each of the six devices. In this way a grand total of 72 counts was recorded for each plate, each individual having made 12 check counts of each plate. In all, 35 individuals have assisted in the counting, using 20 different devices. A total of 37 plates have been counted.

The data reveal a variation from zero to more than 25 per cent in check counts by the same individual on the same plate and an inexcusably larger error in check counts among the different analysts. Results obtained by the most careful individuals indicate that a limit of error of 5 per cent variation between duplicate counts by the same individual is not too low for a standard. In check counts between two individuals a tolerance of 10 per cent can be allowed.

Magnification tends to promote accuracy. Artificial illumination tends to increase counts as well as to promote accuracy.

23. Demonstration of a Simple Anaerobic Culture Dish. ROBB SPALDING SPRAY, School of Medicine, West Virginia University, Morgantown.

A practical and cheap culture dish has been displayed, in which the active reagents, pyrogalllic acid and sodium hydroxide, among others, may be kept separate until after perfect seal is effected. In this dish, in suitable medium, *Cl. tetani*, *Cl. botulinum*, *Cl. welchii* and *Cl. sporo-*

genes have been repeatedly cultured in isolated colonies. These cultures have been repeatedly mixed, and pure cultures recovered.

The dish is simple in design and is a self-contained unit which permits daily examination of cultures. It offers excellent possibilities for photography, as well as for a wide variety of experimental studies on reaction to different degrees of oxygen tension which can be adjusted by diminishing the amounts of the reagents quantitatively. Studies on colony morphology, reaction to differing hydrogen ion concentration, and many other problems may be easily conducted.

It is not designed to supplant the use of the highly developed resources of the anaerobe specialist, but to serve as a simple and useful plating accessory. For the small hospital laboratory, with only limited facilities, this dish should be eminently practicable.

24. A Bacteriological Incubator for Uniform Temperatures with Accurate Control. M. W. JENNISON, Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge.

An incubator for exact control of temperature conditions, consisting of a wooden box lined with 2 inch corkboard, with the incubation chamber, about 18 inches on a side, lined with $\frac{1}{8}$ inch asbestos (Transite). A transite shelf, shorter than the chamber so as to allow for air circulation, is placed 3 inches above the bottom. A 50-watt heating unit, of nichrome wire wound on a strip of transite, is fastened under the shelf beneath which lies a pan of water for obtaining a moist atmosphere. In the center of the back a set of electric fan blades is mounted on a shaft extending through the back, the shaft running on "oilless" metal bearings. A pulley on the outer end of the shaft is belted to an induction motor so that the fan turns at 900 r.p.m. On the inside, at the top, a coil of copper tubing is fastened, through which cold water may be run to obtain temperatures at or below room temperature. Through a hole in the top a mercury thermoregulator is mounted, and connected to a relay operating directly on 110 volts A.C.

Temperatures up to about 60°C. may be obtained, with an operating uniformity and operating sensitivity within $\pm 0.15^\circ\text{C}$., depending somewhat on the specific temperature used. For greater accuracy, an adjustable external resistance may be used in series with the heating unit, to provide maximum and minimum settings.

With the equipment described the apparatus will run continuously for months, with no attention other than an occasional drop of oil on the fan bearings and replacement of water in the humidifying pan.

AGRICULTURAL AND INDUSTRIAL BACTERIOLOGY

1. On the Relation of Refrigeration Temperatures to the Rate of Growth of Certain Specific Types of Bacteria Causing Food Spoilage.

SAMUEL C. PRESCOTT AND P. K. BATES, Massachusetts Institute of Technology and Bacteriological Department, Frigidaire Corp.

A study of specific types of bacteria causing food spoilage has been carried out under conditions of domestic refrigeration in which the temperatures were carefully controlled, and the rates of microbial increase observed.

The organisms studied were isolated from foods undergoing spoilage, and their action observed in pure culture media and in the foods themselves.

It was found that the different organisms of the same general class frequently have a highly specific action in relation to temperature, and also that at the lower temperatures used the relative rates of growth in pure culture are proportional to the temperature increments only for brief periods if at all.

The most important finding is that certain types of spoilage organisms adapt themselves to temperatures assumed to be inhibitive to decomposition processes.

2. The Effect of Discontinuous Refrigeration on Bacteria in Foods.

SAMUEL C. PRESCOTT, PHILIP K. BATES AND H. C. NEEDLE, Massachusetts Institute of Technology and Frigidaire Corp.

To determine the rates at which food deterioration is likely to take place as a result of the discontinuous refrigeration, various foods were kept in electric refrigeration cabinets maintained at constant temperatures of 35°, 40°, 45°, 50° and 60°F. These foods were held in both covered and uncovered containers while at the above temperatures, excepting when they were removed for investigation. Bacterial counts were made on all original samples before refrigeration. The samples were then left in the refrigerators for twenty-four hours and counts were made at the expiration of that period. The foods were then left at room temperature for two hours, at the end of which time counts were again made before replacing the samples in the refrigerators. This procedure was continued for three days. At the expiration of the investigation period, counts were also made on control food samples that had been refrigerated continuously during the experiments cited above, and the foods otherwise compared.

Those foods refrigerated discontinuously showed increases in bacterial growth, the increase being especially rapid at the higher temperatures. Spoilage occurred much more quickly in the discontinuously refrigerated foods, and this was particularly apparent in the case of the covered dishes. In some cases growths increased rapidly even at the lower temperatures for those foods discontinuously refrigerated, while with other foods there was comparatively little change.

3. *Observations on the Bacteriology of Slimy Beef.* SAMUEL C. PRESCOTT, J. F. HALE, AND G. E. WHITE, Massachusetts Institute of Technology.

Beef in cold storage sometimes undergoes a rapid superficial development of microorganisms giving rise to disagreeable odors and the formation of a slimy coating. This was found to be due to a practically pure culture of a bacterium capable of growth at temperatures slightly above the freezing point, especially in the presence of high humidity and insufficient air exchange.

4. *The Reduction of the Number of Organisms in Water as a Result of Freezing in Domestic Refrigerators.* SAMUEL C. PRESCOTT, Massachusetts Institute of Technology, Boston, and PHILIP K. BATES, Frigidaire Corp., Dayton, Ohio.

The study of the numbers of organisms present in ice has occupied the attention of various workers from time to time. With the increasing introduction of domestic mechanical refrigeration, the production of ice in the home has assumed important proportions. The question of the persistence or increase of organisms in ice frozen in mechanical refrigerators has been the object of study by the Food Technology Research Laboratory of the Massachusetts Institute of Technology and the Bacteriological Research Laboratory of the Frigidaire Corporation.

City water was frozen in ice trays of a variety of materials, and quantitative bacteriological determinations by the plate count method were made on the water before freezing and on melted whole ice cubes after freezing and storage for various lengths of time. In one case, large quantities of pure cultures of organisms inoculated into sterile water were used in place of tap water.

All observations indicate freezing reduces the number of organisms in water. Very small numbers of organisms persist for some days, but there is no appreciable increase in organisms over periods up to eleven days.

5. *Some Factors Limiting Bacterial Growth at Higher Temperatures.* E. P. CASMAN AND L. F. RETTGER, Laboratory of Bacteriology, Yale University, New Haven, Conn.

The apparently close relationship between the thermophilic bacteria and the common spore-forming rods, and suggestive literature bearing on oxidation-reduction aspects, led to an attack upon the problem of growth at high temperatures by (1) acclimatization attempts using members of the "subtilis group," and (2) heat lability studies of certain enzymatic oxidation-reduction processes.

The following representatives of the "subtilis group" were selected: *B. subtilis* (Ford), *B. vulgatus* (Ford), *B. mesentericus* (Ford), *B. mesentericus* (Am. M.), *B. subtilis* (Tap w.), *B. cereus* (H. I.), *B. megatherium* (Doryl.), *B. megatherium* (N. Y. U.), *B. prausnitzii* (Am. M.), *B. mycoides* (N. Y. U.).

Acclimatization was attempted by gradual raising of the temperature of incubation during daily transfer of the cultures in beef infusion media. This procedure was followed for approximately a year and resulted in only a negligible degree of success.

The inferiority of nutrient broth to agar slants in the support of bacterial growth at the higher temperatures indicated a special dependence of growth at high temperature upon the oxidation-reduction potential and led immediately to a study of growth in deep glucose infusion agar.

The results revealed considerable diversity with respect to the temperature effects upon various organisms. For example, *B. cereus* (H. I.), *B. prausnitzii* (Am. M.) and *B. subtilis* (F.) showed an inability to grow in the depths of deep agar at the higher temperatures. *B. mycoides* (N. Y. U.), on the other hand, when exposed to its maximum temperature of growth for some time lost its ability to grow at or near the surface of the agar, but retained its ability to grow in the depths. *B. vulgatus* (F.) grew microaerophilically at its maximum temperature of growth.

With this diversity in mind, and in the hope of gaining an insight into the processes responsible for the inhibitory action of heat, certain oxidation-reduction systems were selected for study with respect to their heat labilities. Peroxidase, "para-phenylenediamine-oxidase," catalase and "succinodehydrogenase" were chosen for study.

In testing bacteria for peroxidase, a few drops of bacterial suspension, pH 5.0, were mixed with benzidine and several dilutions of hydrogen peroxide. A blue color indicated the presence of peroxidase. The reagent used in testing for oxidase was para-phenylenediamine which on oxidation in an alkaline medium gives a red to brown color. Catalase

was detected by mixing a drop of bacterial suspension with a drop of 1:50 hydrogen peroxide on a glass slide. Examination for the evolution of bubbles was made macroscopically and, when necessary, microscopically. Methylene blue reduction was studied under anaerobic conditions at pH 7.3 and in the presence of sodium succinate.

The bacterial suspensions employed were prepared by growing the bacteria on meat extract agar for twenty-four hours at 37°C., washing the growth off aseptically with 0.85 per cent saline solution and heating portions of the suspensions through a sufficiently wide temperature range. In order to simulate as much as possible the conditions prevailing in the acclimatization attempts, the suspensions were not washed or shaken very vigorously, and were kept for twenty to twenty-four hours in the incubators used for acclimatization.

The results obtained, although indicating considerable fluctuation in the heat labilities of catalase and oxidase, permitted certain conclusions. Contradictory results obtained with catalase and oxidase seemed attributable to differences in the turbidities of the suspensions used and to the presence of clumps of bacteria.

The heat labilities of the para-phenylenediamine oxidases of *B. subtilis* (F.), *B. prausnitzii* (Am. M.), *B. megatherium* (N. Y. U.) and *B. megatherium* (D.) were found to approximate the maximum temperatures of growth of these organisms. Those of *B. cereus* and of *B. mycoides* (N. Y. U.) varied considerably but as a rule showed a similar correlation.

The catalase of *B. subtilis* (F.) was destroyed at its maximum temperature of growth. In spite of considerable variation, an inhibition of the catalases of *B. mesentericus* (F.), *B. subtilis* (Tap w.), *B. cereus* and *B. prausnitzii* was evident at their maximum temperatures of growth.

The "succinodehydrogenases" of *B. mesentericus* (F.), *B. subtilis* (Tap w.), *B. cereus*, *B. megatherium* (N. Y. U.), *B. megatherium* (D.) and *B. mycoides* were distinctly inhibited at the maximum temperatures of growth of these organisms.

The peroxidases were as a rule heat-stable. An occasional incomplete heat lability was observed with *B. mycoides* and *B. megatherium* (N. Y. U.)

B. mesentericus (Am. M.) and *B. vulgatus* exhibited no correlation between the heat labilities of any of the four systems studied and their maximum temperatures of growth. The former exhibited a resistance of all four systems considerably above the maximum temperature of growth. The production of hydrogen peroxide by *B. vulgatus* may have masked the heat lability of the oxidase which was found capable of withstanding a temperature of 70°C. A similar possibility may obtain

for *B. mesentericus* (Am. M.) although hydrogen peroxide formation by the latter is not very marked.

Five known thermophiles cultivated at the minimum temperatures supporting their growth were also studied with respect to the heat labilities of the four systems. All exhibited a destruction of the succino-dehydrogenase after heating at a temperature approximating the maximum temperature of growth. Oxidase, and in two organisms catalase, exhibited a similar but not so sharply defined relationship.

The results obtained tend to confirm an anticipated diversity in the mechanisms responsible for the inhibition of growth at higher temperatures.

6. *Thermophilic Microorganisms from Imported Canned Shalots (Onions).*

PAUL S. PRICKETT,¹ Bacteriological Laboratory, Mead Johnson and Co., Evansville, Ind.

In an investigation of the bacteriological condition of Italian canned onions which were reported as the cause of an outbreak of botulism, samples of this imported product were examined not only for toxicity but also for their thermophilic flora. An attempt was made to ascertain if any correlations existed between the condition of the container, the pH of the product, the toxicity of the product, and the thermophilic flora. Data were also obtained for comparison with those reported for American canned products.

A total of 127 thermophilic cultures were isolated. Twenty-seven cultures were found to be obligate thermophiles; the remainder were facultative. One hundred cultures, falling into 11 natural groups, were identified as to type. These groups, tentatively identified with previously described species, are listed in the order of their importance based upon the number of cultures isolated in this study as follows: *Bacillus subtilis* Cohn; Cameron and Esty Group 60; *B. thermoalimentophilus* Weinzirl; *B. stearothermophilus* Donk; *B. aerothermophilus* Weinzirl; Cameron and Esty Group 80; *B. calidus* Blau; a miscellaneous group of facultative thermophiles corresponding to groups II, III, IX and X of Tanner and Morrison; a miscellaneous group of obligate thermophiles containing cultures corresponding to *B. michaelisii* Prickett and *B. kaustophilus* Prickett; and an *Actinomyces*. No anaerobic thermophilic types were found.

¹ The material which was used in the investigation herein reported was obtained by the author when formerly employed as Assistant Bacteriologist, Bacteriological Unit, Food and Drug Administration, United States Department of Agriculture.

Little correlation was found between the pH of the cans' contents and the thermophilic types isolated. Only cans normal in appearance were found to contain viable thermophiles alone. When viable thermophiles were found in cans classified as swells or springers, viable mesophilic types were always found associated with the thermophilic types. With one exception, no can was found to contain more than one thermophilic type.

No correlation was found between toxicity of the cans' contents or the presence of the spores of *Cl. botulinum* and the types of thermophiles isolated. A comparison of the percentage of cans containing toxin or spores of *Cl. botulinum* and also containing viable thermophiles with the percentage of all of the cans in which thermophilic organisms were found shows that the former percentage is slightly lower.

Feeding tests with twelve representative thermophilic cultures isolated in this study produced no visible effects in the test animals.

With few minor exceptions all of the thermophilic types isolated from this imported canned food were identified with thermophilic types described as having been isolated from canned foods packed in this country. Furthermore, the few exceptions noted were identified with thermophilic types previously described as having been isolated from other sources in this country.

7. *Time-Temperature-Humidity Relations in Dried Fruit Pasteurization.*

J. A. CLAGUE AND C. R. FELLERS, Massachusetts Agricultural College, Amherst.

Raw, packaged Hallowi and Sayer dates in 7- and 10-ounce paper cartons were subjected to varying controlled temperatures and humidities for different lengths of time. The object was to determine the most effective pasteurizing conditions consistent with a finished product of satisfactory physical properties. Temperatures ranged from 140° to 190°F.; relative humidities from 62 to 100 per cent, and time of pasteurization from twenty to eighty minutes.

Using *Escherichia coli* as the test organism, the following effective minimal processes were determined:

190°F. for 20 minutes at	82% relative humidity or above
179°F. for 30 minutes at	78% relative humidity or above
170°F. for 40 minutes at	72% relative humidity or above
160°F. for 60 minutes at	71% relative humidity or above
165°F. for 60 minutes at	100% relative humidity
145°F.	No effective pasteurizing time under 80 minutes.

Using *Mycobacterium tuberculosis* in tuberculous sputum and the guinea pig inoculation method, packaged dates were effectively pasteurized at all processes exceeding 170°F. at 78 per cent relative humidity for fifty minutes. This process was equally effective against *Escherichia coli*. The physical and organoleptic properties of the dates were actually enhanced by the heat treatment. The average increase in moisture content in pasteurized dates was approximately 2 per cent, not enough to make the fruit excessively moist or to stimulate spoilage by microbiological agencies.

Thermal death point data in both broth and 20 per cent date syrup indicated that *Escherichia coli* (3 strains) were more resistant than *Eberthella typhi* or *Shigella dysenteriae* (Shiga) and equally resistant with *Salmonella enteritidis* and *Salmonella schottmülleri*.

Several hundred samples of pitted and unpitted dates, commercially pasteurized in accordance with these findings, show no coliform organisms, the flora consisting principally of aerobic sporulating bacteria and saprophytic molds.

8. The "Natural" or "Normal" Resistance of Spores of Thermophilic Bacteria. E. J. CAMERON, Research Laboratories, National Canners Association, Washington, D. C.

As one phase of experimental work conducted for the purpose of arriving at process recommendations for canned foods, it has sometimes been the practice to inoculate experimental packs with spores of bacteria originally isolated from foods which were spoiled as a result of under-sterilization. These spores for inoculation were developed in the laboratory and the objection has been raised that the significance of the resultant findings was limited by the lack of information regarding the relative resistance to heat of the so-called "natural" and laboratory-prepared spores.

During the last canning season a study was made of the resistance of spores of spoilage thermophiles as they occurred in equipment and materials used in canning. Sterile nutrient was added to test samples, such as blancher overflow water or sugar. They were then heated in sealed tubes in small quantities (about 2 cc.). There was no manipulation which could conceivably affect the number or resistance of spores originally present in the test material.

It was found that spores occurred under "natural" conditions which were comparable, as regards resistance, to the most resistant developed

in the laboratory. There was also a similarity as regards the range of resistance.

It was also found that sugar may contain spores of such a resistance that, when used with non-acid products such as peas and corn, sterilization can not be accomplished with any practicable process.

9. *The Effect of Concentrated Salt and Sugar Solutions on the Thermo-Death Times of Molds.* G. I. WALLACE AND F. W. TANNER, University of Illinois, Urbana.

Thermo-death times of several species of mold in salt and sugar solutions were studied. *Rhizopus nigricans*, *Mucor mirus*, *Aspergillus niger*, *Penicillium brevicaulis*, *Oidium lactis* and *Alternaria solani* were the molds studied and were suspended in 1, 3 and 6 per cent salt solutions, 10, 25 and 50 per cent sugar solutions, juice from pitted red cherries in syrup and distilled water. Regardless of the suspending medium, the molds were destroyed, with two exceptions, within five minutes at 60°C. *Aspergillus niger* was destroyed within 15 minutes at 60°C., and *Oidium lactis* survived 60°C. for thirty minutes. In general, the molds in salt solution acted in a manner similar to those in distilled water. With *Rhizopus nigricans* and *Aspergillus niger* in sugar solutions, it took longer to kill the organisms at a given temperature than when they were in salt solutions or distilled water. This protective action of the sugar was not indicated in the cases of *Mucor mirus*, *Trichothecium* sp., *Alternaria solani*, *Penicillium brevicaulis* or *Oidium lactis*; in fact it seemed that with *Mucor mirus* and *Alternaria solani* the sugar had the opposite effect.

10. *The Viability of Yeasts in Sucrose Solutions.* F. W. TANNER AND WILLIAM BURROWS, University of Illinois, Urbana.

Numerous statements have been made in the literature that pure cultures of yeasts and related fungi remain viable for a period of years in 10 to 12 per cent solutions of sucrose. The authors have had an opportunity to test the soundness of these statements with some 130 species, constituting the collection of budding fungi at the University of Illinois. Observations were made on two sets of tubes, one in which paraffin was used to seal the tube and the other in which the tubes were sealed with viscose capes. The paraffin-sealed tubes were three years old, while those sealed with viscose capes were only one year old. All tubes had been kept at room temperature which varied between 15°

and 25°C. Twenty-six of 49 pure cultures in 12 per cent sucrose in paraffin-sealed tubes did not grow. Fifty-five pure cultures in tubes sealed with viscose capes did not grow after one year. The medium in the last mentioned set of tubes, had evaporated, which might explain the higher mortality. In some cases, the pure yeast cultures fermented with sufficient vigor to push the paraffin plug from the tube. These data are presented to show that too much confidence should not be placed in this method of keeping pure cultures of budding fungi. Other methods of holding pure cultures are being studied.

11. *Studies upon Bacterial Spores. III. A Contribution to the Physiology of Spore Production in B. mycoides.* B. C. BRUNSTETTER AND C. A. MAGOON, Bureau of Plant Industry, U. S. Department of Agriculture.

The percentage of spores of *Bacillus mycoides* obtained after twenty-four hours incubation at 30°C. in an aerated Difco peptone medium was found to be inversely proportional to the concentration of the medium, within the limits of 0.25 to 2 per cent. In Difco peptone medium of a concentration less than 2 per cent, the percentage of spores formed in twenty-four hours was directly proportional to the rate of aeration. The relative importance of the concentration of nutrients and the concentration of metabolic products in the medium is discussed. The dissociation of *B. mycoides* has been studied and its effect on spore production in liquid media is considered.

12. *Humus as a Source of Energy for Soil Microorganisms, and the Energy Liberated by Their Activity.* F. HESSELINK VAN SUCHTELEN, New Jersey Agricultural Experiment Station, New Brunswick, N. J.

The humus of the soil can be considered the source of energy, as well as of food, for all soil microorganisms except the few obligate autotrophic organisms. It is very hard, of course, to make a distinction between the rôle of humus as food and as a source of energy and it is not intended to suggest the energetical conception as opposed to the material "chemical" point of view. On the contrary it is fundamentally nothing but another point of view of the same process which we call life, and it is hoped that this "other" view will prove to be a welcome and supplementary conception of soil microbiology.

It seems justifiable to consider humus as the source of energy for the soil microorganisms, for the following reasons:

1. In the case of dissimilation, the rôle of the material substance is placed in the background, the energy transformation being paramount.

2. In assimilation more energy is required to build up the body substance than the energy content (combustion heat) of the new growth indicates.

3. Especially in the physiology of microorganisms we have the classical example that the requirements of energy dominate the material requirements, for example the autotrophic bacteria are able to use such widely different substances as H_2 , CH_4 , CO , H_2S , S and other energy containing substances.

In the course of this study the energy value of the humus was determined. The values of the heats of combustion were, as should be expected, not constant or identical, but were found generally to lie between 4.7 and 5.4 kgm. calories per gram. The energy content of the humus may be considered as "metabolizable" energy because of the metabiosis of soil microorganisms.

To determine experimentally the heat liberated by bacteria from humus, it is first necessary to measure the loss of heat of silvered, vacuum bottles the temperature of which is higher than the constant temperature incubator. The soil is put into these thermos bottles, and accumulated heat from microbial humus decomposition is measured. Not the temperatures obtained, but the calories produced are a true measure of the availability of the humus. The temperature of the incubator is kept constant throughout the experiment.

The determination of heat thus produced may be considered a better index of the intensity of microbial life in the soil than other hitherto proposed methods, and gives the following results:

a. Because the experimental conditions provided more oxygen during the early period of incubation, an increased heat production took place during the first fifty hours of the experiment. (One liter of soil may produce in the first fifty hours up to 80 gm. cal. per hour.)

b. After three hundred to four hundred hours the heat production is relatively constant, and often is of the magnitude of two to ten gram calories per hour per liter. These values may serve as a basis for the determination of the availability of the humus and the need of replenishing humus in the soil (manuring and green manuring).

In addition, the writer would like to call attention to the fact that the products of aerobic decomposition in general have a lower energy value (per gram) than the products of anaerobic transformations. In using the necessary discretion this may prove to be a valuable means of orientation as to whether the trend of decomposition in a soil is aerobic or anaerobic.

13. *The Effect of Certain Simple Non-nitrogenous Salts on the Growth of Bacteria in Soil.* H. J. CONN, Agricultural Experiment Station, Geneva, N. Y.

A study has been made of the food requirements of three non-spore-forming soil bacteria when growing in two soils in which these three organisms do not grow naturally. It has been found that growth of the organisms in question may be secured if glucose is added as a source of carbon and energy and some ammonium salt (or amino acid salt) as a source of nitrogen. Similar growth is obtained, however, if one substitutes for the nitrogen certain simple non-nitrogenous compounds. Nearly any potassium salt or potassium hydroxide has this effect; some sodium and calcium salts act similarly, as does sodium hydroxide. Sulfates, carbonates, phosphates and hydroxides of these strong bases have this stimulating effect most noticeably; chlorides, on the other hand, seem to be actually toxic.

The only way the results can be simply explained is to assume that these compounds make the nitrogen already in the soil available to the bacteria. Some of the compounds added, such as the hydroxides and carbonates, might well do this chemically, because of their basic reaction. With some of the neutral salts, however, it is harder to explain the results on a chemical basis, and it is suggested that the effect observed may be due to freeing of the nitrogen from the colloidal complexes in which it is adsorbed.

The data on which this paper is based are to be published shortly as a technical bulletin of the N. Y. Agricultural Experiment Station.

14. *Bacillus radiobacter in Reference to Commercial Legume Inoculants.* LEWIS T. LEONARD, Bureau of Chemistry and Soils, United States Department of Agriculture.

The wide natural distribution of *Bacillus radiobacter*, Beij., its close association with leguminous roots and nodules and its resemblance in certain characteristics to the legume nodule and crown gall organisms suggest the importance of a familiarity with it by those working with the two latter organisms. Since *B. radiobacter* is not described in the better known manuals of determinative bacteriology it is necessary to go to other sources for this information. Löhnis and Hansen consider the macroscopic and microscopic appearance of this organism in detail, and Smith has also added to the rather meager data supplied originally by Beijerinck. By the method of Smith employing crystal violet in dilution water and plating on glycerine-nitrate-soil extract agar it is

possible to obtain very characteristic, raised, smooth glistening colonies with opaque centers and transparent rims from soil, roots, nodules and other materials which commonly harbor *B. radiobacter*.

The presence of *B. radiobacter* has been demonstrated in soils and plant materials from many sources and in the work of examining commercial cultures of nodule bacteria it has not been uncommon to find pure and partially pure cultures of this organism rather than the proper strain of nodule bacteria. One can only speculate as to the exact origin of these contaminating organisms in legume bacteria cultures. The suggestion of Richmond that their occurrence may be the result of an evolution from a mixture of peritrichous and monotrichous nodule bacteria is not confirmed. The close association of *B. radiobacter* with legume nodules and its similarity in appearance to the legume organism especially the peritrichous type would seem to suggest that a mixed colony was selected accounting for the contamination or a mistake was made, the wrong organism being isolated. By the use of Smith's method it has been possible satisfactorily to detect the presence of *B. radiobacter* by plating and this means is suggested to prevent errors in judgment in the identification of the legume organism.

A large number of radiobacter cultures from various sources and different strains of legume bacteria subjected to concentrations of crystal violet ranging from 1 part in 1000 to 1 part in 20,000 indicated that radiobacter possesses in general a greater tolerance for crystal violet than the legume bacteria. It appears from this limited work that the method proposed by Anderson for the isolation of legume bacteria might produce better results in isolating the organism it is expected to exclude.

Twenty-five radiobacter cultures were inoculated into Bryophyllum plants to determine whether they would produce galls. All tests were negative indicating at least a lack of pathogenicity for Bryophyllum. Control inoculations of crown gall organisms gave satisfactory galls.

15. *Floral Changes in the Fermentation of Sauerkraut.* CARL S. PEDERSON, State Agricultural Experiment Station, Geneva, N. Y.

A bacteriological study of eight krauts was made. Three of these were normal and five were inoculated with various pure cultures. Several hundred cultures were studied and grouped. Four species, viz., *Leuconostoc mesenteroides* (Cienkowski) Van Tieghem, *Lactobacillus pentoaceticus* Fred, Peterson and Davenport, *Lactobacillus cucumeris* Bergey, and *Lactobacillus plantarum* (Orla-Jensen) Bergey, have been

found to be the most common organisms in fermenting kraut, although many other types have been found. Many of these are strains of species closely related to those mentioned above.

Pure culture inoculation with *Lactobacillus cucumeris* was found to change the flora of kraut, while the quality of the kraut produced was adversely affected. Pure culture or mixed culture inoculation with other organisms did not materially affect the flora or quality of the product. *Leuconostoc mesenteroides* is largely responsible for the early acidity development. The lactobacilli complete the fermentation. The complete report of this work has been published as Technical Bulletin No. 168, from the New York Agricultural Experiment Station.

16. *The Continuous Lactic Fermentation of Whey.* L. A. ROGERS AND E. O. WHITTIER, Research Laboratories, Bureau of Dairy Industry, United States Department of Agriculture, Washington.

The usual method of conducting an industrial fermentation is to complete the fermentation, replace the fermented medium with new material and repeat the process. Laboratory experiments, previously reported, have shown that if fresh fermentable material is supplied continuously the lactic fermentation will continue at the maximum speed indefinitely.

In the past year the fermentation of the lactose of whey to lactic acid has been carried out on a small commercial scale by a continuous method.

Abnormal fermentation of the supply of whey was prevented by making it distinctly alkaline.

The whey in the fermentation tank which held 4500 pounds was inoculated with *bulgaricus* and held at 44°C.

When the sugar was nearly all fermented, whey from the supply tank was run through the fermentation vat at a rate which permitted the complete, or nearly complete, conversion of the lactose to lactic acid.

Throughout the fermentation the reaction was held between pH 5.0 and 5.8 by feeding in dry hydrated lime.

At a rate of flow approximating the capacity of the fermentation vat every twenty-four hours, the effluent was almost completely free of sugar and gave about 80 per cent of the theoretical yield of lactic acid.

17. *The Importance of Feeds as a Source of Ropy Milk Organisms.* C. N. STARK AND M. J. FOTER, Cornell University, Ithaca, New York.

It is a well-known fact that bacteria which will cause a ropy con-

dition in milk can be isolated from polluted streams, the cow's drinking cup, dust from the interior of the dairy barn, the manure pile and soil. This condition prevails irrespective of whether the dairyman or the dairy plant is having trouble with ropy milk.

The object of the work here reported was to learn the original source of bacteria which cause ropiness in milk. Sixty-three different kinds of grains, seeds, dry hays, green feeds, etc., have been examined. Some of these materials have been tested many times, others only once or twice.

The number of such bacteria found per gram of material examined varied from a few hundred to many thousand. Bacteria which would cause milk to become ropy were found in nine different grain mixtures, corn, hominy, oats, red top seed, buckwheat seed, red clover seed, alsike clover seed, alfalfa seed, raw soy bean meal, wheat bran, cottonseed meal, ground alfalfa hay, chaff, green wheat, and green timothy hay. Negative findings do not prove beyond question that slime producing bacteria are not present.

It is believed that these findings offer some explanation for the presence of a small number of such bacteria in most producers' milk. In the light of data here presented it is felt that feeds may be considered one of the important sources of the bacteria which cause milk to become ropy.

18. *The Origin of the Growth-Restraining Substance in Raw Milk and the Action of this Substance upon an Organism of the Sweet Curdling Type.* HAROLD R. CURRAN, Research Laboratories, Bureau of Dairying, United States Department of Agriculture.

The bacterial growth in the udders of live cows was compared with that occurring in the udders immediately after death and removal of the blood. Preliminary plate counts of the milk from the live animals indicated the normal fluctuations in the udder flora. At slaughter, the blood was rapidly drained from the rear quarters following which the udder was quickly amputated and incubated at body temperature for 4 hours. Plate counts of the milk were made at the beginning and end of the incubation period. The milk from the amputated udder was drawn as completely as possible after the first sample so that the milk obtained after 4 hours represents that which was secreted during this period. In 3 of 5 cows a pronounced multiplication of bacteria occurred during the post-mortem incubation period. The rapidly growing invasive types of bacteria were most affected. The significant relationship

between growth suppression and the blood supply suggests that the blood is in some way associated with the formation of the inhibitory substance in milk.

A sweet curdler, isolated from milk was found to be particularly sensitive to the bactericidal activity of raw Jersey and Holstein milk. This action was present over a period of several months. Coagulation was delayed from three and one-half to six hours in different samples of mixed raw milk. Heating the raw milk in an Arnold sterilizer removed the growth-retarding factor. The marked germicidal activity in this study was confined to Jersey milk. Association with *Streptococcus lactis* did not appreciably influence the development of the sweet curdler and the former organism was only slightly retarded by the bactericidal action of the milk. From these observations it seems probable that a strong germicidal action may be of considerable practical importance when the milk is produced and handled under conditions favoring the entrance and development of these organisms.

19. *Studies on the Eye-forming Organism of Swiss Cheese.* WILLIAM C. FRAZIER AND HELEN U. WING, Bureau of Dairy Industry, United States Department of Agriculture.

The eye-forming organism, *Bacterium acidi-propionici* δ , is usually grown in a peptone-glucose broth to be added to the kettle milk in the manufacture of Swiss cheese. In the past a fixed amount (5 cc.) of this broth culture has been added per 100 pounds of milk, regardless of the age of the culture. Counts on these bottles of eye-former culture have shown that the bacteria vary in number from eight or nine billion per cubic centimeter in five-day cultures down to about six million after a month and about two million after two months. The resistance of these bacteria to the heating which they would receive in the cheese kettle, half an hour at 53°C. with gradual cooling, varies with the age of the culture. Less than one per cent of the organisms of a culture less than 4 days old survive this heating; not over 15 per cent survive of cultures not over 2 weeks old; while up to 50 per cent of two month-old cultures may survive. These facts have been taken into consideration in recommendations for the use of broth cultures of the eye-forming bacteria in Swiss cheese.

A study of the numbers of eye-forming bacteria in Swiss cheese at different stages during the manufacture and ripening has shown that the number of these organisms does not decrease markedly during storage in the cold room (12°C.) for fourteen days. After the cheese is

removed to the warm room (about 22°C.) there is little increase in numbers until the time of the start of eye-formation when the cheese is three or four weeks old. During this time the increase may be from about 9000 per gram to from 50,000 to 150,000 per gram in cheese to which eye-former culture has been added. In cheese to which no eye-former culture has been added, the number of eye-forming organisms three days previous to the first signs of eye formation has been found to be as low as 300 per gram. By the time the eyes have started this number increased to 190,000 per gram. Although the increase in numbers is a little more rapid thereafter, the count does not usually reach a billion per gram until the cheese is about 2 months old, some time after the eyes are completely formed. At this time and later the propionic bacteria usually completely outnumber any other organism in the cheese.

20. *Methods for Determining the Persistence of Certain Important Bacteria in Swiss Cheese.* L. A. BURKEY, Bureau of Dairy Industry, United States Department of Agriculture.

Examinations were made of the cheese at the time it was dipped and at frequent intervals thereafter. The cheese curd was found to contain the larger proportion of bacteria and gave more dependable results than did the expressed whey.

It was found that sodium citrate was more effective in grinding the cheese than either fine sand, sugar or other crystalline substances tried. The technique used is as follows: One gram of cheese is ground for one minute in a sterile mortar with 3 cc. of sterile water at 50°C. (Water cools almost immediately to a temperature slightly above the melting point of fat.) Two-tenths of a gram of sodium citrate is then added and the grinding continued until a smooth even paste is formed. Six and one-half cubic centimeters of sterile water at 50°C. are now added and thoroughly agitated, making a 1:10 dilution of the cheese. This is diluted further for plating. For direct microscopic count 1 cc. of the 1:10 dilution is added to 9 cc. of 50 per cent alcohol (sufficiently alkaline to remain pink with phenolphthalein). This final dilution forms a uniform film on the slide. After it is dried, it is treated with xylol; fixed with alcohol containing a few drops of acetic acid; further fixed by treatment with 2 per cent solution of calcium chloride (for five minutes); and when dry, stained with methylene blue. Decolorization is not desirable.

Litmus milk dilutions, lactose broth Durham tube dilutions, the culture plate method and the direct microscopic count were the methods

used for the determination of the numbers and kinds of bacteria in the cheese.

21. *Comparative Investigations on the Microbiological Composition and the Quality of Butter.* KARL J. DEMETER AND F. X. MAIER, Bakteriologische Abteilung der Suedd. Forschungsanstalt für Milchwirtschaft in Weihenstephan bei Muenchen.

The problem was to find out the possibly existing relations between the score of butter as determined by degustation and the results of the microbiological analysis.

There were examined more than 500 samples of sour cream butter (cream pasteurized at 145°F.) which had been stored ten days at about 38°F. previous to scoring. The scoring was done by two alternating groups of the judging team of the so-called "Bayr. Markenschutzverband für Butter und Käse."

Immediately after scoring the bacteriological examination was made; and it consisted of the following determinations: Mold and yeast count (each one separately), total count on lactose china blue agar (separate determination of acidifying and non-acidifying organisms), total count and number of proteolytic organisms on casein agar. The determinations of the total count on standard agar, of the colon-aerogenes group and of the fat decomposing and the oxidase producing bacteria were abandoned during progress of work as the respective results proved to be in no sense conclusive.

Results: The *mold count* shows only a general relation between high counts and lower grade butter. This, however, holds only for groups and not for individual samples, except those with extremely high counts (more than 50,000 per cubic centimeter). The *yeast count* alone does not give any idea of the quality of butter at all and may be only a means for controlling the proper management of a creamery. The *total count on lactose agar* demonstrates in a general way that the higher the total germ content the lower the grade. The same is true of the *number of the acidifying bacteria*. Naturally there are discrepancies too, concerning individual samples. No relations, however, could be detected, with the content of *non-acidifying* bacteria, except extremely high counts. This holds also for the number of *caseolytic organisms*. The most useful of all determinations proved to be the *total count on casein agar* of Frazier and Rupp. First-class butter, under the conditions of our work, should count less than 1,000,000 per cubic centimeter, never more than 2,000,000 per cubic centimeter. A higher count is prac-

tically always an indication of lower grade butter or at least of the fact that something will go wrong with it in a short time, although a low count does not always warrant a good quality. A butter having a total count of more than 2,000,000 per cubic centimeter on casein agar, should never get a high score, even if the degustation test should be favorable. From these observations, it may be concluded that the casein agar is rather elective for the organisms not wanted in butter.

Between some *typical flavor defects* of butter (rancid, cowy, oily and unclean) and the microflora no definite relations seem to prevail. "Rancid" and "cowy" samples frequently had higher mold counts and higher total counts on lactose agar (acidifiers included) than the "oily" and "unclean" samples. In addition the rancid samples as a whole showed some tendency towards higher total counts on casein agar. No relation could be found between "cowy" flavor and coli-aerogenes content; on the contrary, the respective samples owed their defective flavor least to the so-called contamination.

Furthermore we made investigations on the relation between the microflora of the ten days old butter and the *keeping quality in storage* at 38°F. for eight and sixteen weeks. The total count on the casein agar again proved to be the best means for predicting the keeping quality. The same is true, to a lesser degree, however, with the mold and yeast count and the number of proteolytic organisms.

22. *The Acidoproteolytes (Gorini) in Milk Pasteurization.* COSTANTINO GORINI, Bacteriological Laboratory, Agricultural High School, Milan, Italy.

Bacterial counts do not measure the keeping quality of pasteurized milk, and thus none of the scoring systems takes into consideration one of the most important elements in keeping property, viz., the nature of the surviving microflora, and above all the thermophilic acidoproteolytes (Gorini) which cause the frequently complained of *premature or sweet coagulation* of the pasteurized milk.

MEDICAL BACTERIOLOGY, IMMUNOLOGY AND COMPARATIVE PATHOLOGY

1. *The Effect of Bacterial By-Products on Leucocytes.* ALICE C. EVANS, National Institute of Health, Washington.

The efficiency of the leucocytes which take an active part in the combat between host and invading bacteria is important in determining the outcome of infectious diseases, yet in the literature on the subject con-

ficting statements are found which do not give a clear idea of the effect of bacterial by-products on leucocytes.

The study reported here was planned to show particularly the effect of streptococcal by-products on leucocytes, with parallel tests to show the effect of tetanus and diphtheria toxins on leucocytes for comparison. A modified Neufeld's technic was used, according to which the tests are carried out in small reagent tubes. Streptococci sensitized by the bacteriotropins (stable opsonins) in immune serum were used as "food" for the leucocytes which were obtained from rabbits. In each experiment several lots of leucocytes from a common source were washed in the various test and control materials, and the effect on phagocytic activity was noted. The toxin of *Streptococcus scarlatinae* did not injure the leucocytes. Neither did the tetanus and diphtherial toxins have any effect on phagocytic activity. On the other hand acetic acid reduced the phagocytic capacity of the leucocytes consistently. This is in agreement with the results obtained by the writer in a previous study, in which it was shown that free H-ions are injurious to leucocytes, and that organic acids show a greater toxicity for leucocytes than can be accounted for by the free H-ions in the medium.

2. *Studies on Typhus Fever.* HANS ZINSSER AND M. RUIZ CASTANEDA,
Harvard University Medical School, Boston.

In recent communications we have reported that the Rickettsia bodies of Mooser can be obtained in large numbers in the peritoneum and in organs in which they have hitherto not been found, both in rats and in guinea pigs in which the resistance has been lowered by injections of benzol. In such animals Rickettsia have been found in sufficient amounts in the peritoneum to permit washing them free of cells and protoplasm. With such washed Rickettsia the typical disease has been produced in guinea pigs; and guinea pigs recovered from infection with the washed Mexican Rickettsia have been found immune to injections of unconcentrated virus of both Mexican and European typhus strains. These experiments have seemed to us to prove the etiology of the Rickettsia bodies in both varieties of typhus, and our confirmation of Pinkerton's findings of typical Rickettsia in the tunica vaginalis of European typhus guinea pigs if examined early has further strengthened this conviction.

In the present communication we report that we have found a still better method for obtaining large amounts of Rickettsia by feeding guinea pigs and rats on vitamine-free diets until they are in very poor

condition, then inoculating them in the usual way with typhus virus and continuing the diet. Such animals have invariably yielded considerably increased numbers and distribution of *Rickettsia*, and in some of them enormous amounts in the peritoneum and sometimes in the pleura. The basis of such experiments was an epidemiological one, the tremendous mortality in times of famine being considered.

The practical purpose of the experiments was to obtain sufficient amount of *Rickettsia* material for vaccine purposes, since in a previous paper we have reported experiments which indicate that active immunization may be possible with *Rickettsia* killed by formalin, provided a sufficient concentration of the organisms can be obtained.

In the present paper we also report upon a series of experiments which, though not one hundred percent successful, demonstrate unambiguously that active immunization with formol-killed *Rickettsia* material will sometimes immunize completely and when it does not do this will modify the disease distinctly in the direction of greater mildness. These experiments are the beginning of a larger scale immunization which will be continued with the more concentrated *Rickettsia* material that the diet method has placed at our disposal.

We have succeeded in obtaining multiplication of *Rickettsia* in Maitland cultures but do not believe that this method is at the present time equal for vaccine production to the obtaining of the organisms in concentration in animals of diminished resistance.

3. *Presence of Fusiform Bacilli and Spirochetes in the Mouths of a Group of Young Adults.* MARGARET HOTCHKISS, The New York Homeopathic Medical College and Flower Hospital.

From 161 subjects (26 women and 135 men) preparations were secured by swabbing the necks of the lower molars near the gums. The material was spread on a clean new slide, fixed with methyl alcohol, stained by Gram's method, and examined for fusiform bacilli and spirochetes. The subjects were all active young adults in the twenty- to thirty-year age group. The condition of the teeth varied but gave no indication of the microscopic findings which were:

a. Spirochetes only.....	1
b. Fusiform bacilli only.....	20
c. Fusiform bacilli and leukocytes.....	5
d. Spirochetes and fusiform bacilli.....	40
e. Spirochetes, fusiform bacilli and leukocytes.....	56
f. No fusiform bacilli or spirochetes.....	39
All preparations showed gram-positive diplococci.	

Although 96 subjects gave a microscopic picture commonly associated with "Vincent's angina" only two of those questioned gave any history of previous clinical symptoms. The subjects were all connected with the same institution, 78 since September, 1930, and 83 since at least September, 1929; nineteen were resident. Thus, in the group of healthy individuals which formed this study, spirochetes and fusiform bacilli were common mouth inhabitants. If these organisms are as prevalent elsewhere, the demonstration of such a combination by microscopic examination would seem of doubtful diagnostic significance.

4. *Do Saprophytic and Secondary Bacteria Occurring in the Respiratory Tracts of Domestic Fowl in Infectious Trachitis and in Health Produce Agglutinins in the Blood?* CHARLES S. GIBBS, Massachusetts Agricultural Experiment Station, Amherst.

Sixty-one cultures consisting of staphylococci, escherichia, micrococci, streptococci, alcaligenes, eberthella and pasteurella were isolated from the respiratory tracts of twelve fowls suffering from acute infectious trachitis and of ten healthy fowls not immune to infectious trachitis. Blood was drawn from the axillary veins of these fowls, allowed to clot, and the serum secured for agglutination tests. The agglutination tests were made in dilutions extending from 1:8 to 1:2040. The range of titer in the tests was found to vary from 0 to 1024 without showing any specificity other than what could have been classified as natural or spontaneous agglutination. While great variations existed among different serums in agglutination titer, the results demonstrated that saprophytic and secondary bacteria in the respiratory tracts of domestic fowls were of little importance in producing agglutinins in the blood.

5. *The Occurrence of Diphtheroids in Routine Blood Cultures.* LUTHER THOMPSON, Mayo Clinic, Rochester, Minnesota.

Method: Immediately upon withdrawing the blood from the patient, 2 cc. are added to 30 cc. of glucose brain broth in a 1- by 7-inch tube. At the end of fourteen days, or sooner if growth is apparent, a Gram stain is made, and 0.1 cc. of the sediment is inoculated into a tube of soft brain agar (0.3 per cent agar) in $\frac{5}{8}$ - by 8-inch tubes. These are in turn incubated for fourteen days unless growth appears earlier.

Approximately 600 cultures have been studied. Diphtheroids have not been observed to appear in cultures before the third or fourth day in this series. Between 2 and 3 per cent of all cultures negative after two days have later shown a growth of diphtheroids.

6. *Multiple Antibody Production.* C. A. BEHRENS, Purdue University, Lafayette, Indiana.

Rabbits fail to respond to restimulation beyond a certain degree. The sequence of the antigens used, apparently, has no marked effect upon antibody production.

The tendency is toward the formation of hemoagglutinins rather than hemolysins after 3 or 4 different kinds of red blood cells have been injected. The non-specific development of hemoagglutinins for rat and horse are especially pronounced.

The restimulating effects of sera, particularly rat serum, are marked.

Evidence of Forssman's antigen is present in beef, horse and chicken serum.

Depending upon the allergic condition of the animals and the precipitinogens employed, the production of non-specific precipitins for human, guinea-pig, horse and rat serum is demonstrable.

7. *A Comparison of Methods for Determination of Potency as Observed on a Group of Horses Immunized against Diphtheria.* GEORGE F. LEONARD, Biological Laboratories, E. R. Squibb and Sons, New Brunswick, N. J.

In a group of horses which were immunized for the production of diphtheria antitoxin, the potency of each was determined by three different methods.

1. *The Government Official Test for determining the units of antitoxin.* This is an accurate method, but when a number of individual horses are to be tested it requires a large number of guinea pigs, as well as much time and labor.

2. *The intracutaneous test.* Mixtures of a standard diphtheria toxin and dilutions of the serum from trial bleedings were injected intracutaneously into guinea pigs. When the toxin was not neutralized a reaction was seen as in the Schick Test. The advantage of this test is that a number of dilutions can be tested on the same guinea pig.

3. *The flocculation test.* This test was made by mixing in a test tube dilutions of the serum to be tested with a standard diphtheria toxin. Flocculation took place first in the tube in which there was an exact neutralization of toxin and antitoxin. This method has the advantage of simplicity and economy.

Trial bleedings were made on each horse at monthly intervals, and a study of comparative potency tests was continued for a period of two

years. The results of the three methods of testing have been plotted on charts.

The charts show that there are some discrepancies in the different methods of testing. However, for practical purposes in the production of diphtheria antitoxin, either method could be used in testing the potency of the individual horses.

For economy and accuracy the intracutaneous test seems to be the method of choice.

8. *Studies on the Precipitin Reaction. III. Further Studies on Conditions Influencing the Formation of Precipitates.* SELMA GOTTLIEB AND CORNELIA M. DOWNS, Division of Sanitation, Kansas State Board of Health, and the Department of Bacteriology and Immunology, University of Kansas.

1. Further studies have been made on the precipitin reaction, using crystalline egg albumin as precipitinogen.

2. The salts used represented mono-, di-, and trivalent cations (including salts of the alkali and alkaline earth groups) and also represented mono-, di-, tri-, and tetravalent anions.

3. The specific precipitate, regardless of the salt present when it was formed, showed the same solubilities in weak acids and weak bases.

4. The salts which inhibit formation of the precipitate do not dissolve the precipitate when it has once been formed unless they are acid or alkaline by hydrolysis.

5. Salts of the heavy metals give non-specific precipitates which interfere with a determination of their effect on the precipitin reaction.

6. Salts have no effect on the production of precipitation below the zone of precipitation although they may intensify precipitation when antigen and antibody are present in proper proportions.

7. The effect of a given salt is the same whether the precipitinogen is crystalline egg albumin or horse serum, but in some cases is intensified when the purified antigen is used.

9. *The Influence of Antigen-Diluents on the Intensity of Flocculation in the Precipitation Test for Syphilis.* EMIL WEISS, Department of Bacteriology, Pathology and Preventive Medicine, Loyola University Medical School, Chicago, Ill.

A study was made in order to determine whether the saline in the precipitation test for syphilis (Kahn's and author's methods) can be replaced by a more effective diluent. Various salts and acids in increasing

concentrations were used. Sodium sulphate in saline and sulphasalicylic acid in watery and saline solution were found to be more satisfactory than normal saline. This was the case with Kahn's and the author's methods, regardless of whether Kahn's or the author's antigens were used.

10. *Bile as an Antigen in Serum Diagnosis of Syphilis.*¹ NATALE COLASI, New York.

The author has used plain alcoholic and cholesterinized extracts of ox-bile, instead of the ordinary extracts of normal organs, as antigen in the Wassermann reaction for syphilis. From the results of 125 comparative tests with known normal, one plus, two plus, three plus and four plus sera, and with known negative and four plus cerebrospinal fluids, it is deduced that:

1. Ox-bile possesses the antigenic principle common to extracts of normal organs. It can be used, therefore, as an antigen in the Wassermann test for syphilis.

2. Aqueous solutions, plain alcoholic and cholesterinized extracts of ox-bile are equally reactive as Wassermann antigens as are similar solutions and extracts of normal organs.

3. Sheep bile is hemolytic to ox and sheep red-blood corpuscles and cannot be used, therefore, as antigen in the Wassermann reaction, at least when the anti-sheep or anti-ox hemolytic system is used.

4. Alcoholic and cholesterinized extracts of ox-bile react similarly as antigens with normal, one plus, two plus, three plus and four plus sera and with negative and four plus spinal fluids as do corresponding extracts of normal organs.

5. Plain alcoholic and cholesterinized extracts of ox-bile, properly prepared and preserved, are recommendable for use as antigens in the diagnosis of syphilis by the Wassermann reaction.

11. *Cultural Characteristics of Neisseria gonorrhoeae.* R. B. H. GRADWOHL, St. Louis, Mo.

Work was undertaken because of dissatisfaction in clinical and medico-legal practice with the usual method of diagnosis by Gram stain of smears. There is a very meager description of the cultural characteristics of *Neisseria gonorrhoeae* in the literature.

Media described are not always successful in everyday practice.

¹ Experimental data from a thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at New York University.

There is a demand for a rapid successful differential medium. Such a medium was described by the author.¹ The present paper deals with experiences with this method, together with colony description from enlarged photomicrographs. Control methods are described. Practical application of the method particularly in gynecological practice is discussed.

12. *The Effect of Urine on Hemolytic Streptococci.* G. H. ROBINSON AND F. A. TAYLOR, Wm. H. Singer Research Laboratory, Pittsburgh, Pa.

In the routine bacteriologic examination of catheterized urine it was observed that such streptococci as were obtained were invariably of the non-hemolytic type. There seemed to be two possible explanations viz.: (1) hemolytic streptococci infecting the urinary system had lost their hemolytic properties or (2) hemolytic streptococci were destroyed by the renal secretions.

A large number of hemolytic streptococci from various conditions were grown in sterilized normal urine. Growth was found to be very poor. By transferring from urine to broth and then to urine, cultures were passed through 15 generations in urine. No loss of hemolytic power was observed.

Non-hemolytic strains from whatever source remained alive in urine with no apparent loss of vitality for more than 6 weeks. For hemolytic strains the length of life in urine was less than one week.

In order to determine the factors responsible for these results, various fractions of urine are being studied for their effect on hemolytic streptococci.

13. *Serologic and Bacteriophagic Relationships in a Group of Fecal Streptococci.* PEARL KENDRICK AND HARRIETT C. HOLLON, Michigan Department of Health, Western Michigan Division Laboratory, Grand Rapids.

Data are presented for about 100 strains of fecal streptococci and 50 other strains. They have been studied with respect to their agglutinability by an immune serum specific for a culture of fecal streptococci and their susceptibility to lysis upon first contact with a filtrate strongly lytic for the homologous culture of the immune serum.

Under the experimental conditions, a definite parallelism exists

¹ Journal Amer. Med. Assoc., 1926, 87, 242.

between the serologic and bacteriophagic reactions of the fecal streptococci studied.

14. *Studies of Tularemia Infection in Rabbits and Guinea Pigs. I.*

CORNELIA M. DOWNS, Department of Bacteriology and Immunology, University of Kansas, Lawrence.

A study of tularemia infection in rabbits and guinea pigs has been made in normal animals and in animals injected with killed cultures of *B. tularensis*. The organism used was isolated from a typical case and has been cultured for two years on cystine-glucose-blood agar. The minimum lethal dose of the strain used was approximately ten organisms per gram of rabbit weight. In normal rabbits the organisms invaded the blood stream after seventy-two hours in sufficient numbers so that a small amount of rabbit blood killed guinea pigs with typical lesions. In rabbits immunized by long injection of killed cultures, the organisms invaded the blood stream in ninety-six hours.

The skin at the site of the injection was uninflamed in the normal rabbits and guinea pigs while in the immune rabbits twenty-four hours after injection of the culture the skin at the site of injection was red and inflamed. The lesion at the death of these animals in seven to ten days was well defined and indurated at the edges but healing in the center. A series of guinea pigs which had received injections of killed cultures when given a dose of living organisms, gave a caseous and indurated skin lesion invariably over the site of the injection.

Animals injected into the heart with a large number of organisms died in two and one-half days with slight lesions. Animals scratched with about 0.1 cc. containing approximately the same number as was injected in the heart died in seven days with well developed lesions. The lesion at the site of injection was much more inflamed and indurated than in normal pigs. Normal pigs receiving the same amount in the skin and heart died in four days with practically no reaction at the site of injection.

The difference in the local reaction between the normal and immunized rabbits and guinea pigs undoubtedly is indicative of an immune reaction. The injection of crystalline egg albumin into the skin as given by Dienes produced a good titer of precipitins against albumin but nothing resembling a tuberculin reaction. The antibodies circulating in the blood stream seem not to give a very good indication of immunity, as animals showing agglutinins of 1:5000 die with typical lesions in only a slightly longer time than the normal animal. It is common to find

agglutinins in the serum in a dilution of 1:500 to 1:1000 at the time of the death of the animal.

15. *Electrophoresis Studies on Lactobacillus acidophilus and Lactobacillus bulgaricus Cultures*. R. H. WEAVER, University of Kentucky, Lexington.

The electrophoretic charges (P.D.) carried by the organisms of 31 authentic cultures of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* were measured, using the Falk cell (slide type). After being grown in milk, followed by two serial transfers in tomato juice broth (Kulp) the organisms were thrown out of suspension by centrifugation, washed three times and examined suspended in distilled water.

With three exceptions cultures labelled *L. acidophilus* carried lower charges than those labelled *L. bulgaricus*. Individual cultures showed a variation from one extreme to the other rather than two distinct groups. Two of the exceptions were among intermediate cultures. Definite records of positive implantation experiments are available for 7 of the 8 *L. acidophilus* cultures bearing the lowest charges. Similar records of negative implantation experiments are available for 3 of the 6 *L. bulgaricus* cultures carrying the highest charges. No unquestionable records are available for the other cultures.

Fermentation experiments failed to separate the cultures into two groups.

16. *Serologic Studies with L. acidophilus (Moro) and L. bulgaricus (Grigoroff)*. MARGARET F. UPTON AND NICHOLAS KOPELOFF, Department of Bacteriology, Psychiatric Institute and Hospital, Columbia Presbyterian Medical Center, New York City.

The purpose of this investigation was two-fold: (1) to determine the serologic properties of the "X" or rough strains, and the "Y" or smooth strains, of *L. acidophilus* (Moro) of intestinal origin and of *L. bulgaricus* (Grigoroff). Agglutination and absorption tests were carried out with serum from immunized rabbits. (2) The attempt was made to force dissociation of the smooth into the rough strain of *L. acidophilus* (Moro). Rough and smooth strains were cultured in ten per cent homologous serum.

The results, with a few cultures, indicated that there were marked serologic differences between rough and smooth strains respectively of *L. acidophilus* (Moro). The same is true of the rough and smooth strains of *L. bulgaricus* (Grigoroff). On the other hand there is evidence which

shows that the smooth strains of *L. acidophilus* (Moro) and *L. bulgaricus* (Grigoroff) are serologically identical. Some of the rough strains of these two species also have agglutinins in common.

It would appear that there is a closer relationship between the smooth strains of *different* species of these lactobacilli than between the rough and smooth variants of the *same* species.

Heating the antigens at 100°C. for either ten minutes or one hour failed to exert any influence on the agglutination reactions.

Rough strains of *L. acidophilus* (Moro) dissociate spontaneously into smooth variants. Recently dissociated smooth strains revert to rough strains on frequent transfers in broth or when resting on agar slants for long periods of time. We have never seen smooth strains spontaneously dissociate into rough variants.

How can we differentiate *L. acidophilus* (Moro) from other lactobacilli or *L. bulgaricus* (Grigoroff) or further, how can we establish the therapeutic efficiency of either the rough or smooth strains of *L. acidophilus* (Moro)? It is suggested that the capacity to transform the human intestinal flora be the sole criterion.

17. *Types of Br. abortus Isolated from Human, Bovine, Porcine and Equine Sources.* WAYNE N. PLASTRIDGE AND J. G. McALPINE, Storrs Agricultural Experiment Station, Storrs, Connecticut.

Two hundred sixty-five strains of *Br. abortus* isolated from human, bovine, porcine and equine sources have been identified by Huddleson's dye plate method and by the glucose utilization method of McAlpine and Slanetz.

Strains of human origin: 129 strains isolated from cases of undulant fever occurring in the United States and Europe were classified as follows: porcine type 66, bovine type 63.

Strains of bovine origin: Of 60 strains isolated in the United States from cattle, 8 were found to be porcine type and the remainder bovine type. Fifty of the strains received from Germany, Austria and Hungary were examined. Forty-eight were classified as bovine type, while 2 failed to give results sufficiently clear cut to justify their classification as either the bovine or porcine type.

Strains of porcine origin: 22 strains of porcine origin were identified as typical *Br. abortus* (porcine type).

Strains of equine origin: All of the 4 strains of equine origin possessed the differential characteristics of the bovine type.

The results obtained by Huddleson's dye plate method were in close agreement with those obtained by the glucose utilization method.

18. *Copper Sulphate Medium for the Isolation of Br. abortus (Bang) from Milk.* I. FOREST HUDDLESON, Central Brucella Station, Bacteriological Section, Michigan State Experiment Station.

In order to isolate *Brucella abortus* (Bang) from raw market milk it is necessary to use the gravity cream layer. If streptococci are present in the market milk, they are also concentrated in the cream layer. When the cream is cultured they grow out rapidly and lower the pH of the medium. *Br. abortus*, if present, will not grow. Streptococci fail to grow out on beef liver infusion agar, pH 6.6, containing gentian violet in a dilution of 1:200,000 and copper sulphate solution in a final dilution of 1:2000, while *Br. abortus* (Bang) is not inhibited.

19. *The Agglutination Titre of Milk as an Indicator of the Presence of Bact. abortus in Milk.* HERBERT L. GILMAN, Veterinary College, Cornell University, Ithaca, N. Y.

The increasing number of cases of undulant fever which have apparently been contracted from the drinking of raw milk has led to efforts to reduce the possibilities of human infection from this source. Pasteurization of milk is perhaps the safest and easiest method, but is not ideal by any means. The elimination of *Bact. abortus* infection from herds supplying raw market milk cannot be applied at once.

The present investigation entailed the taking of the blood titre of the cow by the agglutination test together with the milk titre from the individual quarters. Milk from each quarter was then injected into guinea pigs and later examined for lesions due to *Bact. abortus*. The purpose was to see if there was any correlation between the agglutination titre of milk and the presence of the organism in it.

The conclusions reached were: (1) The milk from all four quarters of 68 cattle was examined for agglutinin content, and injected into guinea pigs for evidence of *Bact. abortus* infection. (2) *Bact. abortus* was not recovered from milk showing agglutinins under positive at 1:80, nor from an animal with a lower blood titre than positive at 1:320. (3) *Bact. abortus* was recovered from 59 per cent of the milk from quarters showing agglutinins in dilutions of positive at 1:80 or higher. (4) Tentatively, it may be assumed that quarters showing agglutinins

positive at 1:80 or above are actively infected with the Bang bacillus and may eliminate the organism at any time. Quarters showing agglutinins under 1:80 only in rare instances contain or eliminate the organism. (5) Composite milk samples from all four quarters are unsuitable for use in the agglutination test.

20. *Lethal Temperatures for Br. abortus with Special Reference to Pasteurization.* C. M. CARPENTER AND R. A. BOAK, School of Medicine and Dentistry and the Strong Memorial Hospital, University of Rochester, N. Y.

One hundred twenty-two samples of raw market milk were collected from 67 villages and 2 cities and examined for *Br. abortus* infection. Twenty and four-tenths per cent of the samples showed evidence of *Br. abortus* infection when 2 cc. of the cream from each sample was injected subcutaneously into guinea pigs.

Two hundred and five samples of pasteurized milk and cream collected in 38 cities and 12 villages and examined for evidence of *Br. abortus* by guinea pig injection were negative.

Temperatures of 142° and 145°F. for twenty and thirty minutes on the most virulent strains of *Br. abortus* were found satisfactory for pasteurizing milk.

21. *An Investigation of the Antiseptic and Germicidal Efficiency of Pyridium on Various Organisms.* BERNARD E. PROCTOR, Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge.

The antiseptic and germicidal efficiency of Pyridium, (phenylazo-alpha-alpha-diamino-pyridine hydrochlorides) was tested, using *Escherichia coli*, *Eberthella typhi*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Corynebacterium diphtheriae* and *Pseudomonas aeruginosa* as test organisms.

The agar cup plate method as described by Reddish was used for the testing of relative antiseptic power. Transfers from areas showing no growth on the agar cup plates were made to determine the presence or absence of living organisms in those areas. Pyridium gave evidence of antiseptic action with respect to each of the test organisms used. Further experiments demonstrated germicidal powers in respect to *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Corynebacterium diphtheriae*

22. *The Bactericidal Efficiency of Certain Organic Peroxides.* BERNARD E. PROCTOR AND NICHOLAS A. MILAS, Department of Biology and Public Health and Research Laboratory of Organic Chemistry, Massachusetts Institute of Technology, Cambridge.

A series of organic peroxides, synthesized by one of us, in the Department of Organic Chemistry of the Massachusetts Institute of Technology, have been investigated with respect to their efficiency as bactericidal agents. Several of these compounds have been previously unknown.

The tests used in this investigation were modifications of the Hygienic Laboratory Method.

The germicidal powers were found to vary somewhat according to the chemical compounds from which the peroxides were derived.

23. *Merthiolate as a Preservative for Biological Products.* H. M. POWELL and W. A. JAMIESON, Lilly Research Laboratories, Indianapolis, Ind.

The usual phenoloid preservatives used in bacterial toxins, vaccines and other labile antigens, as well as in immune serums, have a marked tendency to cause loss in potency or effectiveness. A more nearly ideal preservative would appear to have a wide field of usefulness.

Merthiolate, sodium ethylmercuri thiosalicylate, a new organo-mercury compound which is highly soluble and non coagulating in action as well as relatively atoxic, has been tested as a preservative for such biological products. Under severe conditions, as well as the usual cold storage conditions, Merthiolate causes practically no demonstrable damage to antigens such as diphtheria toxin and toxoid, tetanus toxin, various bacterial hemotoxic fractions and the more labile bacterial cell proteins, and to various antitoxins. The antibody response to Merthiolate-killed and preserved bacterial cultures has proved much greater than that following experimental use of heat-killed and phenoloid-preserved cultures.

24. *The Effects of Alkaloids on Bacteria.* O. B. WILLIAMS, University of Texas, Austin.

This study of the effects of alkaloids on bacteria included observations on the inhibition of growth, on motility, cell morphology and pigment formation. Soluble forms of ten alkaloids have been tested with a number of species for inhibition of growth. Considerable differences in toxicity for different species have been observed. Concentrations of

the more toxic alkaloids which permitted growth did not restrain motility although cell morphology may be quite appreciably affected. The development of pigment may be delayed or entirely prevented by concentrations of alkaloids which still permit growth.

25. *The Phenomenon of Local Skin Reactivity to Various Bacterial Culture Filtrates. A Review.* GREGORY SHWARTZMAN, Laboratories of the Mount Sinai Hospital, New York City.

In contrast to *B. diphtheriae*, *Cl. botulinum*, *Cl. tetani*, scarlet fever and erysipelas streptococci, *B. dysenteriae* and a few others, the majority of pathogenic bacteria were not hitherto shown to possess any true toxins. The inability to demonstrate such toxins is due to the fact that the laboratory animals respond irregularly and non-specifically to the injection of filtrates of the majority of pathogenic bacteria. The immunological phenomenon about to be described tends to demonstrate the existence of soluble neutralizable toxins in a great variety of microorganisms, (i.e., typhoid, paratyphoid, coli, dysentery, hemorrhagic septicemia, hemoglobinophilic groups, meningococcus, gonococcus, a few strains of streptococci and pneumococci).

If the skin of a rabbit is injected with a small amount of a bacterial filtrate a slight erythema or no reaction follows. However, if 24 hours later the same rabbit is injected intravenously with the filtrate of the same or another bacterium, four to five hours after the intravenous injection there appears an extremely severe hemorrhagic necrosis at the previously injected skin sites. The factors determining the local skin reactivity are termed "skin preparatory factors" and those responsible for the local injury following the intravenous injection are called "reacting factors."

Normal rabbits vary in their susceptibility to the phenomenon. There is no apparent relationship between the primary effect of intradermal injections and the intensity and size of the local reaction following the intravenous injection. The optimum incubation period necessary for the skin preparation is twenty-four hours. The reactivity disappears completely in 48 hours. The reactivity is induced by a single injection. The reaction is severe and appears rapidly. The second injection must be made by the intravenous route. Numerous substances of non-bacterial origin (histamine, nitroglycerin, india ink, casein, horse serum, egg albumen, turpentine, etc.) and culture filtrates and vaccines of some microorganisms fail to induce skin reactivity to potent bacterial filtrates and also fail to elicit the phenomenon to themselves. The

skin preparatory and reacting factors are found only in certain bacterial cultures, fluctuate in potency, are filterable and are obtained in maximum concentration in young cultures under conditions of insignificant cell autolysis. There is a close parallelism between the phenomenon producing potency and the lethal effect of bacterial filtrates. The potency of skin preparatory and reacting factors can be accurately titrated. Both sorts of factors can be specifically neutralized by immune sera and, therefore, it is possible to measure quantitatively the neutralizing antibodies of a given serum. Bacterial variation has a profound influence upon the antigenic structure of the factors involved. The phenomenon can also be produced if filtrates from entirely unrelated microorganisms be used for the skin preparatory and reacting factors, respectively, provided the filtrates be sufficiently potent.

On the basis of the facts thus far obtained it is concluded that the phenomenon described is not in the nature of local anaphylaxis. It demonstrates a state of induced susceptibility to neutralizable soluble toxins from a great variety of microorganisms. There appear to be various applications of the phenomenon, namely: to immunological studies on the course of infectious diseases; to measurement of antitoxic potency of therapeutic sera and the development of antitoxic sera; to studies on the pathogenesis of disease; to etiological studies on certain diseases, etc.

*26. Biochemical Studies of Bacterial Derivatives. XV. Skin Reactions in Man: A Comparison of Tuberculin O.T., Human Tubercle Bacillus Protein MA 100 and Timothy Bacillus Protein MA 100.*¹
F. M. HUNTOON, E. H. FUNK AND HARRY WHITE, Glenolden, Philadelphia and White Haven, Pa.

This report is part of a general problem designed to determine the biochemical nature of the chemical derivatives of the tubercle bacillus and allied organisms.

The immediate problem is to determine the nature and frequency of the intradermal skin reactions produced by the MA 100 proteins derived from the human tubercle bacillus and the timothy bacillus by identical techniques. These are compared with a standard tuberculin O.T. as a control. The points to be noted are: (1) The similarity in appear-

¹ The material employed was furnished by the Mulford Laboratories; the testing was done at the Pennsylvania and Jefferson Hospitals, Philadelphia and the White Haven Sanatorium, White Haven, Pa.

ance of the reactions produced by the three materials; (2) the marked difference in size of dose between the two proteins to produce reactions of comparable size; (3) the percentage of reactions obtained in tuberculous individuals and those presumably nontuberculous.

1. The reactions with the MA 100 proteins are not to be distinguished from those obtained with Tuberculin O.T.

2. A dose of 0.0005 mgm. of the human tubercle bacillus protein MA 100 gives a reaction of equal size to that obtained with 0.01 mgm. of timothy bacillus protein MA 100 and 0.00001 cc. of the Standard Tuberculin O.T.

3. The percentage of reactions cannot be given at the time of writing as the work is still in progress.

27. *Studies of the Properties of a Bouillon Filtrate of the Gonococcus.*

L. T. CLARK AND N. S. FERRY, Parke, Davis and Company, Detroit, AND A. H. STEELE, Wayne County Training School, Northville, Michigan.

Cultivation of *Mic. gonorrhoeae* in a medium peculiarly adaptable to organisms of this type produces, within a few days at ordinary incubator temperature, a luxuriant growth characterized by a heavy pellicle floating on a clear fluid with a very slight sediment.

The filtrate from this growth exhibits properties similar in nature to a soluble or an extracellular toxin. These properties are recognized by the ability of the filtrate, in high dilutions, to produce typical local skin reactions in a large percentage of individuals and to stimulate the formation of a serum in an animal, which, *in vivo* as well as *in vitro*, will neutralize this toxic substance and prevent the inflammatory reactions which follow intradermal injections of the filtrate.

Controlled by products of autolysis more or less endotoxic in nature, this filtrate manifests properties entirely foreign to products resulting from cell destruction.

The undiluted filtrate is toxic for laboratory animals following intraperitoneal injections and will produce local skin reactions in the rabbit and guinea pig following intracutaneous injections.

These inflammatory skin reactions on the human and the animal as well as the intraperitoneal fatalities in the animal may be prevented by neutralizing the toxin in the filtrate with its homologous antiserum.

While these reactions may be allergic in nature the evidence appears to favor the view that they are toxic.

28. *Studies on Pollen and Pollen Extracts. VI. Rabbit Skin Reactions in Quantitative Estimation of Activity of Pollen Extracts.* H. W. CROMWELL AND MARJORIE B. MOORE, Swan-Myers Company, Indianapolis, Ind.

A modification of Ramsdell's trypan blue skin reaction was used to demonstrate immediate skin reactions to pollen in immune rabbits. The reaction may be extremely sensitive, in several instances positive results being obtained with the extract from $\frac{1}{1,500,000}$ gram of ragweed pollen per cubic centimeter. If proper precautions are taken, a graded series of reactions with a definite end-point may be obtained with the different pollen dilutions. Two or more extracts showing differences in activity when tested simultaneously on one animal show similar quantitative differences on others.

29. *A Study of the Action of Short Electric Waves on Several Biological Substances with Special Reference to Diphtheria Toxin.* WACLAW T. SZYMANOWSKI AND ROBERT ALAN HICKS, Western Pennsylvania Hospital, Pittsburgh.

Highly potent diphtheria toxin is found to be diminished in strength by the action of short electric waves, 1.9 and 3.76 meters, at a frequency respectively of approximately one hundred and fifty-eight million and eighty million cycles per second. The greatest attenuation produced so far was obtained by six hours irradiation. As demonstrated by skin tests in guinea pigs, the toxicity of the irradiated sample was reduced so that 50 skin test doses was found to give a skin reaction which was less than the reaction elicited by one skin test dose of the control toxin. This result was obtained under conditions which eliminated the heat factor.

A film of toxin is exposed between the condenser plates in a resonating circuit which carries a current up to five amperes. This film is constantly cooled between the walls of concentric tubes by means of a chilled fluid circulated in the central tube. Benzol, acetone and distilled water have been tried as cooling liquids and the effectiveness of the radiation is found to be altered by the character of the central cooling fluid. The results obtained are only very roughly proportional to the time of the exposure.

The diminution of potency in diphtheria toxin is produced by this radiation at temperatures which do not alone affect the toxin. This fact indicates that there is an effect produced by radiation which is independent of the heat factor.

The immunizing properties of the attenuated toxin have not been investigated.

The action of similar radiation on the precipitating antibody for pneumococci is completely negative. If the heat effects are eliminated, the sterilization of milk and the protection of experimental animals against streptococcal infections by such radiation is also completely negative thus far in the advancement of this work. Similar studies of related biological substances are giving less certain results.

RECIPROCAL EFFECTS OF THE RELATIONSHIP OF BACTERIOLOGY AND MEDICINE¹

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Received for publication December 23, 1930

In order to limit the scope of this investigation it will be necessary to depend upon that faculty of the mind by which it can be aware of an entirety while examining an element. An immanent sense of the relations existing through all science is requisite for any brief inquiry into the effect of one biological science upon another. Therefore, with an appeal to that comprehensive quality of understanding, I shall try to discuss the reciprocal influences in the relationship of bacteriology and medicine, particularly medical bacteriology and experimental medicine.

The most conspicuous outcome of the relationship of bacteriology and medicine has been the advantages shared by each from this association. In these days, it is unnecessary to make a new detailed catalogue of them. The air is vibrating with the reassuring voice of preventive medicine. Processions of pages in clear print pass before the eyes of all, advertising the exploits of "microbe hunters" and "riders of the plagues," who have "conquered disease." Although there is a good deal of fiction in popular writings on the romance of preventive medicine, and although there is some mud along with good water in the torrent of scientific journalism, informed persons will agree with the statement of Sir William Osler that during the period since Pasteur's papers in 1857 on lactic acid fermentation, "more has been done to determine the true nature of disease than in all the time that had passed since Hippocrates and Celsus." Some,

¹ Presidential address, delivered before the Society of American Bacteriologists at its thirty-second annual meeting, Boston, Massachusetts, December 30, 1930.

knowing how many keen observations on the nature of infectious disease there were before Pasteur, will question the selection of this date as the beginning of an epoch. It seems evident, also, that without Pasteur or Koch a germ theory of disease would have sprung from the observations of many others. It is true, however, that the work of Pasteur and his successors filled a great gap "in our knowledge of the organic cycle and natural history." "This was perhaps the most rapid advance of descriptive knowledge in the history of science." The influence of this growth during the past seventy-five years constitutes a bacteriological era in medicine. During this time, bacteriology has given medicine the knowledge of at least the approximate causes of infectious diseases, has shown how to reduce the incidence and stop the spread of many of them and has discovered how to cure a few. With this aid from bacteriology, the great advance of public health and hygiene has been made. Aseptic surgery, based on bacteriological knowledge, has not only reduced pain and postponed death, but has also been the means for making discoveries in physiology. Bacteriology has provided medicine and biology with a storehouse of interesting and useful substances, together with investigative methods of great power. With an approximately correct theory, bacteriology rescued medicine from floundering in a morass of etiological superstition, and aided in the liberation of the spirit of man by giving him a new sense of ability to know and control some of the hitherto invisible forces influencing his destiny. During these years, medicine, the "mother of the sciences," has had a solicitude verging upon maternalism, for this youngest of the sciences. Medicine has provided bacteriology with much of its material for observation and test, with ancient methods of scientific procedure and with stimulating interest. From medicine, bacteriology has received rich subsidies in money and in maintenance. These medical contributions to the physical establishment of bacteriology are everywhere apparent in hospitals and great institutes for medical research. A very important aid to bacteriology derived from medicine has been the thousands of pages devoted by medical journals and books to the publication of the writings of bacteriologists. Be-

hind these generalizations there is an infinitude of instances giving evidence of the reciprocal benefits in the relationship of bacteriology and medicine. The two sciences are inseparably associated and their continued united attack upon infectious disease should advance each science, contribute to biological knowledge and render increasing service to animals and man.

Behind this triumphal front, however, the allied sciences have had some difficulties in their relationship. Investigators in medical science have expressed disapproval of the influence of bacteriology upon medicine and bacteriologists have found the development of their science hampered by the attitude and demands of their medical associates. These hindrances may be grouped in three categories: disadvantages arising from an intellectual attitude, disadvantages due to inadequate biological training and lack of general or special scientific competence, and disadvantages created by practical arrangements. If these reciprocal hindrances in the relations of bacteriology and medicine have the force attributed to them by some authors, they are apt to be more influential than the reciprocal helps upon the development of these sciences. It will be profitable to examine them in the hope of aiding the untrammelled growth of the individuality of each science and of prospering their coöperative enterprises.

In discussing these reciprocal disadvantages, it is best to abandon the personification of science. The endowment of a science with the qualities of a living organism permits useful generalizations and avoids circumlocutions, but has the danger of leading to muddled thinking. All the observations and their formulation in general laws, which we call science, are the product of the human mind. They are, therefore, the product of the sense perception and rationalization of an individual or groups of individuals. One or more individuals become dominant and the science they create is an expression of their activities. In this case, in discussing the influence of an intellectual outlook, it is necessary to attempt to examine the outlook of Pasteur, Koch and Claude Bernard. The discussion of disadvantages arising from inadequate biological training and lack of scientific competence must be based upon the effect of those characteristics of

individuals. The disadvantages due to practical arrangements obviously are related to the effects of subsidies given for definite purposes, of physical locations and equipment and the influence of demands for immediately practical results.

It has been said by Professor L. J. Henderson that the "intellectual climate" was made unfavorable for the science of experimental medicine by the attitude of Pasteur. Pasteur's outlook was the "outlook of a chemist and in him the will was more important than the reflective intellect." A contrast is made here with the view-point of his contemporary, Claude Bernard, whose "outlook may be described as biological and philosophical," providing the biological and rational intellectual climate favorable to the growth of experimental medicine and general physiology. It seems as if the debate between Pasteur and the ghost of Claude Bernard, which rose from a posthumously published notebook to say that Pasteur's vitalistic theory of fermentation had been destroyed by Bernard's discovery of zymase, were being continued by the mythical figures which personify bacteriology and experimental medicine. Later Buchner proved that Bernard was right, but Pasteur in the life overcame the ghost and the influence of Claude Bernard upon medicine was overwhelmed by the authority of Pasteur. Medicine entered upon a period "when bacteriology was the dominant influence . . . and the prevailing activity was somewhat unintellectual." In another place, Professor Henderson writes: "The science of pathology is notoriously backward and non-rational. . . . Upon the appearance of the bacteriological period in the history of pathology an intellectual decline, as I think it may be fairly regarded, set in. In accordance with the simple views of Pasteur, the specific virus came to be regarded as the cause of each disease, and this view could hardly be questioned during the time of the rapid progress in the discovery of microorganisms. The hypothesis is roughly correct, but it has obscured the organic character of disease and the mutual dependence of the many variables which must be taken into account if the state of the patient is to be understood." The meaning of these criticisms seems to me to be that Pasteur limited his interest to the effects produced by

microorganisms upon materials and animals, while Claude Bernard was interested in the more fundamental mechanisms of the phenomena of life. Pasteur seemed to regard bacteria as chemical reagents. Bernard was searching for the ultimate nature of the underlying physico-chemical processes which brought about these final states. In a general way, Pasteur, with his notions that fermentation was "*la vie sans air*," and that in infectious disease "*life prevents life*," had a tendency to reintroduce into science the idea of life as a hidden cause. Not much emphasis has been placed upon the influence of the vitalistic philosophy of Pasteur. But it seems to me that it is a point of view that explains Pasteur's conviction that he had forever settled the question of spontaneous generation, and his failure to advance the technic of study of the bacteria themselves. Duclaux, who knew him well, said that physicians were right in regarding Pasteur as a chemist. In commenting on Pasteur's delighted interest in Metchnikoff's theory of immunity due to the activity of phagocytes, he says "the idea was the idea of a biologist and naturalist, it had not occurred to Pasteur." Pasteur seems to have had a conviction about the fixity of species through variations in form which served him well in some studies of fermentation, but was not altogether an advantageous view-point to transmit to medical bacteriologists when linked with the concept of constancy of action. These and other examples which might be cited, tend to substantiate with contemporary evidence the modern criticism that Pasteur did not have as broad a biological horizon as Claude Bernard. In the use of rational theory these men are said to have differed extremely. Looked at in retrospect, Pasteur's scientific life seems to have had an admirable unity, as if it were the "logical and harmonious development of one and the same thought." He seems to have proceeded from crystallography to fermentations, from disorders of fermentations to disorders of animals, and from the spread of yeast in the soil and air of a vineyard to the manner of the spread of the agents of infections, all culminating in the hypothesis of the germ theory of infectious disease. Koch, following him, made the apparently rigid experimental tests of verification which fixed upon medical

thoughts Pasteur's "simple view" that the specific virus caused the disease. On the other hand, the history of Pasteur's many investigations, seems to indicate that he did not always proceed through rational theorizing to the steps which led to these great discoveries. Many have admitted an element of opportunism in his work. His chief problems were those presented to him by practical necessity. Once in the problem, however, his amazing insight and prepared mind allowed him to seize upon the significance of the phenomena he observed and to draw far reaching conclusions from them. Duclaux says that "in his heart, Pasteur was indifferent to theories and asked of them only that they suggest experiments to him." For the followers of Pasteur, who had far less insight than he, this indifference to theorizing was to give a decidedly empirical quality to the medical study of infectious disease. Claude Bernard, on the other hand, constantly called attention to the value of imagination and preconceived theory in experimental investigation. He writes: "To be worthy of the name, an experimenter must be at once a theorist and practitioner." "An able hand without a head to direct it, is a blind tool; the head is powerless without its executive hand." Claude Bernard placed so much emphasis upon rational theorizing that there is danger that some of his disciples may forget his equal emphasis upon the necessity for the theorizer to be completely master of the art of establishing experimental fact. There are many examples of Claude Bernard's indifference to particular theories, though not to theorizing, once his experiment was under way. His eye, unclouded by preconceived notions, saw the phenomena as they occurred. Several of his important discoveries were made while "he was looking for something else." Like Pasteur, he had a prepared mind and insight, and seized upon the essential significance of his findings. The difference between the two men seems to have been that without care for theorizing Pasteur expected accidentally to discover facts, while with intense devotion to theorizing, Claude Bernard deliberately discovered unexpected facts. The course of experimental medicine associated with the infectious diseases in the past seventy-five years has exhibited both these characteristics of empiricism and

rational theorizing. Brilliant discoveries have been made under the guidance of the germ theory of disease, but medicine also has continued to be "driven into action and from numberless ventures into the realm of empiricism has gained useful information." Meanwhile, the study of the mechanisms of these phenomena along the lines of physiology and general physiology were delayed by the inrush of bacteriological discoveries. The use of these facts in the making of etiological diagnoses has been a substitute for study of the patient as a whole. Perhaps this influence is much less now than it was some years ago. It has, however, been potent and still exists. To the extent that attention has been diverted from the study of patients to be concentrated too narrowly upon the supposedly specific microbe, Pasteur's influence has been detrimental to the progress of a biological and rational science of experimental medicine.

In fact, the influence of bacteriology upon medicine has been one of the reasons for raising a question about the right of medicine to be called a science. Recently the British Medical Research Council has asked: "is there a science of experimental medicine of which the actual material for study is the human patient, or is scientific work by the physician or surgeon limited to the application in his art of scientific results worked out elsewhere in the laboratory and delivered to him for use?" The question has been answered in several ways. After casting up the account, the Medical Research Council concluded that there is a science of experimental human medicine and cites the work of Sir Thomas Lewis as proof. Lord Moynihan has answered vigorously that there has always been a science of medicine and cites the contributions of modern surgery as the evidence of an active hominal experimental medicine in the present day. In addition he recalls that the inductive method was established by Hippocrates, who drew a general truth from a multitude of singular examples, and that to Galen, who made true experiments of verification, we owe the deductive method and the submission of isolated questions and hypotheses individually to tests. The scientific method, established by these great physicians of antiquity, has remained with medicine throughout its evolution, surviving periods of superstition and

the dominance of authority. Some time ago, Professor L. J. Henderson, noted that Bacteriology "had resigned its leadership of the medical sciences" and that with the turn of the century "the movement which Claude Bernard had forseen" made itself felt. "The result has been a remarkable increase of experimental investigation and of rational theorizing in the clinic." He finds that physiology and especially general physiology are being recognized and pursued as foundations of medical science and that with this recognition "we have now definitely entered upon the epoch of experimental medicine."

The growth of the science of bacteriology has in a like manner been adversely influenced by the outlook of two groups of individuals. One of these has been the host of medical practitioners and pathologists whose dominance in the domestic economy of schools, hospitals and institutes has confined the stream of medical bacteriology in narrow courses of utility. Their point of view may in part be attributable to the influence of Pasteur or of their interpretation of the elements of empiricism and practicality in Pasteur's work. It is in part attributable to the extraordinary influence which diagnosis and some forms of therapy have had upon medical science and medical practice. It is in a large part attributable to the perfectly justifiable belief that the support of bacteriology in such institutions was warranted only on account of the practical service rendered. The second group of dominant persons, whose outlook seems to me to have had some retarding influence upon the growth of the science of bacteriology are the founders themselves, Pasteur and Koch and their followers. I hope I may not seem irreverent in criticizing the masters or to have a shallow appreciation of their vast contributions when I call attention to this apparent paradox in the hindrance which their influence has placed upon the science they caused to flourish. Pasteur himself advised such criticism of authority, though he did not welcome it. It seems to me that what has been said about the effect of the somewhat restricted biological horizons and non-rational tendencies of these great men upon medicine may be repeated with slight modification in connection with their influence upon bacteriology. Both Pasteur

and Koch discovered many of the fundamental biological processes of the microorganisms, but I believe that so much of the influence of these leaders of bacteriological research and thought came from the practical utility of their studies that they themselves were influenced to regard the bacteria from a narrow viewpoint. Both Pasteur and Koch owe some of the steps leading to their eminence to the fact that their work was done in relation to men in the intensely anthropocentric environment of man's physicians. Through them medical bacteriology became a dominant influence in bacteriology, and its tenets were accepted generally. Pasteur's semi-vitalistic conceptions and concentration on the effects produced by bacteria have been preserved as dogma by followers under his authority. The strict notions of bacterial monomorphism so useful to Koch when he was untangling mixed cultures became invested with a sanctity which has delayed until recently the recognition of variation of form and the study of its significance. For a long time there was little of the physico-chemical approach to the study of the phenomena presented by bacteria.

Medical bacteriology, and probably other specialties in bacteriology, have been responsible for raising a question like the one asked of medicine: "Is there a science of bacteriology of which the chief material for study is the bacteria?" The question is a conditioned reflex in a medical bacteriologist who converses in the descriptive terms of clinical bacteriology, who yearns for the statement of a generalization which will satisfy many of the questions answered so superficially by the Pasteur-Koch germ theory of disease and who repeats with crossed fingers the glib phrases of the functional jargon of immunology. Those who have to do with securing provision for bacteriology in universities and other institutions know that this question is asked by others. It is inherent in a statement of Lord Balfour in his preface to the recently published English "System of Bacteriology," where he writes "though the bacteria are the subject matter of bacteriology, they do not, in and for themselves, constitute its main interest." An affirmative answer to this question might be derived from the titles of certain books called "Text-books of General Bacteriology."

The contents, however, show that the generalized aspects treated within them are the effects of all sorts of bacteria on all sorts of substrates, animals and man, milk and tobacco, and many other things. Throughout them there is a sprinkling of information about the bacteria, but the chief interest is in what they do. My experience has been that any bacteriology other than medical bacteriology is regarded as general bacteriology, no matter how specialized. The relation of bacteria to higher organisms and the utility of bacteria in the arts and industries have so greatly engrossed attention that evidence of the science of bacteriology and need for its extension have received too little notice, even among bacteriologists. This inner science seems to me to be the study of the bacteria according to the program of Claude Bernard for experimental medicine—a physico-chemical study of all the phenomena presented by these organisms in themselves and in their relations to their environment in inert substrates or in animals and plants. There is abundant evidence that this science exists. The material of bacteriology is unique. No other group of biologists have in their hands so varied an assortment of manageable protoplasm, and with it unique contributions of far reaching significance have been made, such as the cultivation of cells in artificial media. Its concern in the fundamental conditions and processes of life has been continuous ever since the days of the controversy which removed spontaneous generation from the field of urgent problems to that of a philosophical necessity. Its attention to the question of the relation of organization to life has been constant from the time when Pasteur introduced notions of a sort of atomic structure of the minute viables to the most recent studies on filterable viruses. Many bacteriologists, better biologists though less influential than some dominant men in the science, have known that the bacteria in and for themselves constitute a main interest. To them, as well as to others engaged in dispensing the services of bacteria, we owe contributions to this general science of bacteriology. But it must be admitted that other groups of biologists and physiologists, taking advantage of the extraordinary serviceability of the wide range of vital phenomena presented by the

bacteria, have done more than bacteriologists to advance the fundamental knowledge of fermentation, respiration and the energetics of cells. Their contributions, along with others, go into the literature of this science and contribute to great new volumes on the biochemistry and physiology of the bacteria. Publications like the *Journal of Bacteriology*, books in this country and abroad on life cycles of bacteria and even biologically conceived text-books on medical bacteriology and immunology all testify to the fact that an extraordinarily active period of "investigation and rational theorizing" has begun in the science of bacteriology.

The effect of inadequate biological training and lack of general scientific competence of workers in medical bacteriology has been to foster most of the narrowing easily understood conceptions of Pasteur and Koch and to cause neglect of or even failure to see phenomena of wide significance. These troubles have come about from the type of training the physician has required to make him an expert diagnostician and the chief kind of competence he has considered necessary in his bacteriological coolie. The disadvantages of this to medicine have been recognized and attempts to remove them are being made by the provision of "academic careers" for medical biologists. The medical bacteriologist, who has often been a physician, has carried into his study of bacteria the methods of his unbiological medical training and has been forced by circumstances or inclination to substitute satisfaction in diagnosis for investigation into fundamentals. Even if we exempt from criticism here the masters of the science and numerous eminent scientists working in institutes of medical research, there remains a mass of inadequately trained bacteriologists that gives color to the whole. In 1908, Neisser, had these in mind when he noted that many who had the title of bacteriologist were what he called only "plate-streakers, germ-counters and smear-strainers." He made a plea that the bacteriologist should be an expert adviser to the physician. He cannot, however, be a fundamentally sound expert adviser unless he has a biological outlook and the inclination and opportunity for extended study of the bacteria themselves. Without such an outlook as this

there will be a continued outpouring of the accounts of incomplete and unrelated observations, forming a literature which will introduce as much confusion into the general science as it has into taxonomy. Without a broader point of view and greater scientific competence, medicine and bacteriology will follow each other around in a narrow circle of etiological dogma.

The very practicality of bacteriology has created difficulties of physical establishment for the science. As the physician is content to drop the subject as soon as the bacteriologist hands him the diagnostic report, so those who have provided money, quarters and equipment for bacteriology have often limited this physical assistance to satisfaction of the requirements for practical results. Again there are notable exceptions, and again there is a preponderance of establishments in which the study of bacteria in and for themselves can be conducted as a side line only under cover of other immediately useful bacteriological services. In many places where material for the investigation of fundamental problems is available, no provision for research has been made and it is difficult to obtain funds, equipment, space and men to carry on such studies. In many medical schools there are separate independent establishments for bacteriology. From these, it is true, many important contributions to general bacteriology have been made. But these departments have to serve the needs of medical education and apply most of their resources to the investigation of limited problems in medicine with little more than passing attention to the fundamental phenomena presented by the bacteria. These departments have a sort of academic and budgetary independence; they rarely provide for intellectual independence. In other schools, bacteriology remains in a subordinate place in the departments of medicine or pathology which have been themselves in spiritual bondage to the younger science. All these arrangements have been dictated by necessity and justified by practical rewards. This continued limitation to the narrow field of immediate utility, however, does not seem to be in the best interest of either the science of bacteriology or the science of experimental medicine.

In this discussion of the reciprocal hindrances in the relation-

ship of bacteriology and medicine, there have been scattered bits of evidence that each science was overcoming the difficulties. There is indeed, abundant evidence that the point of view of dominant groups in each science is becoming more and more biological and rational. If bacteriology had a leading position among the medical sciences in the days of its infancy, it will assuredly be prominent, as Almquist has remarked, in the time of its maturity. But a one-sided influence is not desirable or necessary in the relationship between these two biological sciences. Each is fundamentally concerned with a broad investigation of the phenomena of life. Each has its special material for this study and both use the material supplied by the complex of the association of microorganism and animal in infectious disease and other states. It seems to be recognized that both sciences will be served best by employing the methods of physiology and general physiology in the attempt to discover the mechanisms of these microbes and macrobes. Many differences between the aims of bacteriology and medicine have been composed and indications have been given of the way to secure a vigorous science of experimental medicine and a science of bacteriology founded on the bacteria. It seems likely, now, that Claude Bernard and Pasteur are sitting in amicable conversation in adjacent elysian chairs as they used to sit at the sessions of the French Academy before the publication of the famous notebook. If they are in agreement, we shall make great progress by guessing their thoughts.

BACILLUS DIPHTHERIAE IN ITS RELATIONSHIP TO BACTERIOPHAGE

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Received for publication, June 12, 1930

Among the more thoroughly studied bacterial species must be included the diphtheria bacillus, and yet, if we judge from contemporary evidence, its behavior, both as a biological entity and as an agent of infectious disease, is but imperfectly understood. With each new report on its attributes, data are presented bearing on mutation phenomena, dissociation, reversion processes, cyclogeny, and like changes, which all point toward the conclusion that fixity in type and in properties involves a concept no longer tenable.

The profound effect of external environmental conditions upon such characters as morphology, virulence, and toxigenesis is clearly shown by the work of Gins and Jermoljewa (1928-9), to mention but a single instance. That other, and perhaps more subtle, features may equally influence the organism and its behavior is apparent from the results herein to be recorded, bearing as they do upon the relation of bacteriophage to this organism. Study of the relation of *B. diphtheriae* to bacteriophage has been limited (Blair, 1924, Fejgin, 1925), and the observations on record offer nothing of great importance beyond demonstrating the fact that with this organism also the phenomenon of bacteriophagy may occur. It is obvious that, could well-defined bacteriophagy be demonstrated with *B. diphtheriae*, this organism would offer possibilities for study not presented by the majority of bacterial species. Questions of pleomorphism, of fluctuations in virulence, of modifications in toxigenic power, and on the constitution of toxin and, in particular, on the nature of the toxin-antitoxin reaction, might be approached through new avenues.

With some of these possibilities in mind, sporadic attempts have been made in this laboratory, since 1921, to discover or derive a race of bacteriophage active with respect to *B. diphtheriae*, but until this past year no clear-cut results had been obtained. The problem has always been complicated, since there was no way other than mere chance of bringing into contact a potent bacteriophage filtrate and a susceptible bacterial strain. Both of the essential components were, in effect, unknown quantities, and with no assurance that the bacterial strain under examination was sensitive, it was always impossible to affirm that a filtrate did or did not contain bacteriophage. Recently, however, renewed attempts made in connection with other studies upon the diphtheria group of organisms have yielded results of some interest.

The susceptible strain of *B. diphtheriae* which has served throughout the experiments here reported was derived under the following circumstances.

A boy, aged four and one-half years, entered the New Haven Hospital on September 17, 1929, with an admission diagnosis of mild scarlet fever, although at this time there were no throat symptoms. The only point worthy of mention in connection with the previous history is that the child had received smallpox vaccination on the 6th of September, 1919, and on the 15th a cutaneous eruption developed which spread somewhat during the 16th and 17th. The temperature was but slightly elevated. After admission to the Hospital, the rash disappeared and by the 20th of September had completely faded. No further symptoms of any kind developed until September 29, when a swelling of the cervical glands was noted. This was not associated with a temperature reaction or with pain. On the 7th of October the temperature suddenly became elevated, the glands became still further enlarged, and the throat was definitely inflamed. On the 11th a membrane developed and throat cultures revealed diphtheroid-like organisms in direct smears. Later reports on the virulence of the cultures taken at this time were "virulence questionable." The child received antitoxin, and suffered no further complications, although additional cultures which were taken and with which repeated virulence tests were made were recorded as being positive and virulent. After the requisite period of negative cultures the boy was discharged from the Hospital on the 4th of November.

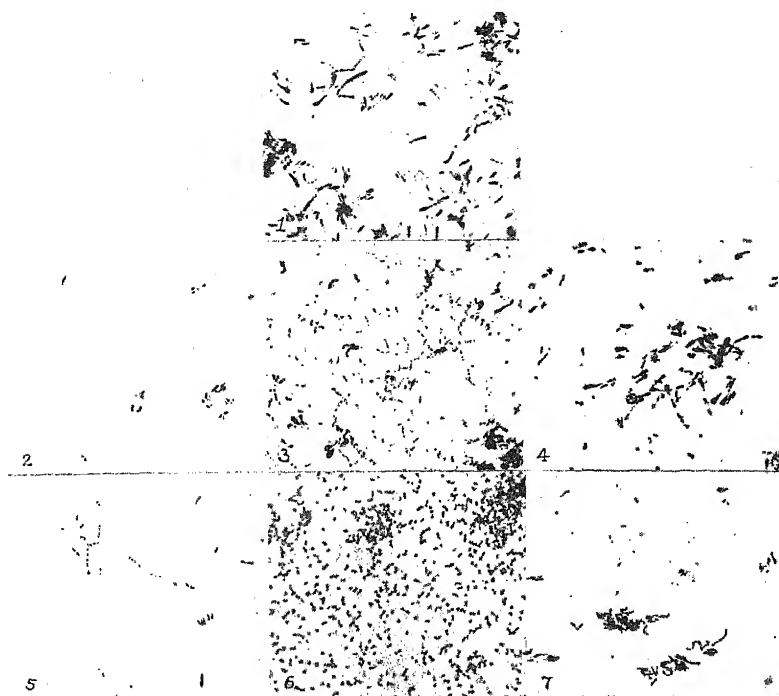


FIG. 1. THE ORIGINAL WRIGHT CULTURE AS FIRST ISOLATED FROM THE PATIENT

FIG. 2. RESISTANT ORGANISMS OF THE LARGE COLONY COMPONENT OF THE WRIGHT CULTURE

FIG. 3. SUSCEPTIBLE ORGANISMS OF THE SMALL COLONY COMPONENT OF THE WRIGHT CULTURE, AS THEY APPEARED AT THE TIME OF ISOLATION

FIG. 4. SUSCEPTIBLE ORGANISMS, WITH ADDED BACTERIOPHAGE, JUST PRIOR TO THE BEGINNING OF LYSIS

FIG. 5. THE SUSCEPTIBLE STRAIN AS IT APPEARS SEVEN MONTHS AFTER ISOLATION

FIG. 6. THE SUSCEPTIBLE STRAIN ON BESREDKA'S EGG MEDIUM

FIG. 7. THE SUSCEPTIBLE STRAIN TAKEN FROM BESREDKA'S EGG MEDIUM AND GROWN UPON SERUM-AGAR

The culture which has served for the experiments herein recorded was a virulent organism derived from this case, and in its original form, is designated as the "Wright" strain. When obtained it possessed no distinctive characters and was of the Wesbrook C type (fig. 1). The alterations which it has subsequently undergone will be discussed later.

The bacteriophage which served in the initial experiment to effect bacteriophagy with this Wright strain was obtained from a sewage filtrate. The raw sewage in question had previously served as a source of supply for bacteriophage races showing a variety of potencies. For present purposes this sewage, after filtration, was added to nutrient broth in the proportion of nine parts of sewage filtrate to one part of a ten-fold concentrated broth. This material, in 50 cc. quantities, was inoculated with 0.2 cc. of broth cultures of each of nine freshly isolated strains of diphtheria bacilli. Four such preparations were made. The object, of course, in such multiple inoculations was to offer any bacteriophage that might be present in the filtrate an opportunity to become enriched if any of the nine strains contained susceptible organisms. Of the four bottles inoculated, only one exhibited, after incubation, any inhibition of growth, and here it was incomplete.

The material from this bottle was filtered (in all filtration experiments Chamberland L3 or L5 filters were employed), and, after demonstration that the filtrate was sterile, it was distributed throughout a series of tubes, and these were in turn each inoculated with one of the nine cultures previously employed, in order to ascertain with which of the nine strains the filtrate was most active.

This titration showed that if the inhibition previously observed was bacteriophagy, culture Wright was the organism most susceptible, and it was upon the basis of this examination that this culture was selected for all further studies. That the result obtained with this filtrate and culture Wright was in effect bacteriophagy was proved by subsequent tests in which it was shown that serial transmission of the phenomenon could be effected indefinitely with the titer of the bacteriophage maintained at a dilution of

10^{-6} , and also by the demonstration of plaque production upon solid media.

In the course of the initial studies upon plaque formation it was noted that colonies of two types were frequently present in cultures of this Wright strain which had grown for periods of forty-eight hours or longer. The first of these was a large, rough, freely-growing colony, resembling in many respects diphtheria cultures which have been for a long time under artificial cultivation. Morphologically, the bacteria composing colonies of this sort were of the Wesbrook C type, but presented a marked tendency toward extreme pleomorphism with the occurrence of a variety of somewhat bizarre "involution forms" (fig. 2).

The second variety of colony was small and fine, presenting no distinctive features other than its minute size. Morphologically the organisms were of the C and C² types. They were beaded and very definitely presented a polar distribution of the deeply-staining areas (fig. 3). Subsequent study has indicated that these two types of colony and the morphologically distinct organisms composing them bear definite relationships to various factors entering into bacteriophage phenomena with this organism.

When this apparent dissociation was observed the two types of colony were separated and the cultures thus derived were tested for their susceptibility to the bacteriophage filtrate. It was then found, and this has subsequently been confirmed many times, that the large colony derived from this Wright strain is not susceptible to bacteriophagy. The small colony, on the contrary, manifests, just so long as it maintains its own distinctive features, a complete susceptibility to lysis.

For the studies in mind it was desired to have available several cultures of diphtheria bacilli of diverse derivation, all susceptible to bacteriophage, and, consequently attempts were made to adapt this bacteriophage to other diphtheria strains. To this end repeated contacts between filtrates of known potency for the Wright strain and the other cultures were made. In most instances no evidence whatever of such an adaptation could be disclosed, while in others results of a questionable nature were obtained. In no instance, with this series of cultures, did a complete adaptation occur. Failing, therefore, to adapt this race of bac-

terioophage to other cultures it seemed reasonable to resort to the alternative expedient and seek other races of bacteriophage.

At that time the question of the homogeneity or heterogeneity of diphtheria bacilli as regards bacteriophage was an open one. As sources for such races recourse was had to a variety of materials. Two other sewage filtrates were tested, stool filtrates from a diphtheria patient at different periods during the disease, stool filtrates from a convalescent diphtheria case, blood from a patient convalescent from a streptococcus infection in which the blood culture showed organisms of the diphtheroid type; stool, urine, and throat washings from a diphtheria carrier; stool filtrates from an untreated, fatal case of diphtheria; throat washings from normal persons as well as stool filtrates from normal individuals, and culture filtrates of the first isolation of the Wright strain.

Without entering into details as to the success obtained with these various materials, since the question of distribution of diphtheria bacteriophage merits consideration elsewhere, it may be stated that races of bacteriophage exhibiting a greater or less degree of potency were recovered from many of the above sources. In all, 11 races of bacteriophage were secured. During the meantime additional strains of *B. diphtheriae* had been isolated directly from field cultures received from the Connecticut State Department of Health, and with these, 36 in number, contacts were made with each of the 11 filtrates.

It was felt that such a titration might serve to yield additional susceptible races of *B. diphtheriae* and might also aid in resolving the question of homogeneity. For reasons which will appear below, these tests were largely unsatisfactory, but they did serve to demonstrate that among the 36 cultures under test no other example of a completely susceptible strain was present. It may be added that although several hundred cultures have since been examined we have yet to find a strain comparable in susceptibility to the Wright culture described above.¹ Thus, all of the work

¹ Since this manuscript was submitted for publication a second susceptible strain, isolated from a field culture, has been obtained. For a period after its isolation this culture behaved in every respect like the Wright culture. It also developed a resistance as did the Wright strain, but unlike the latter it has thus far failed to yield susceptible derivatives.

which has been done has been conducted with this susceptible derivative of the original Wright strain, and the very fact that in all of the tests only one susceptible organism has been employed militates against drawing broad conclusions from the data here offered. Nevertheless, under the limitations imposed by these conditions, the results which have been obtained are of such clear-cut character and are so suggestive that this preliminary report may be justified.

In presenting this material two aspects of the question may be considered, since each is of importance, although from different points of view.

First, the peculiar attributes and behavior of this susceptible Wright strain merit attention since the potentialities of this individual culture may be of significance to an understanding of the cyclogeny of *B. diphtheriae*. The second feature worthy of comment involves a consideration of the properties of field cultures of diphtheria bacilli in their relationship to this particular Wright strain and to the various bacteriophage preparations active upon it.

The Wright strain of *B. diphtheriae* has now been under daily observation for a period of over eight months and during this time on various occasions it has exhibited rather striking variations in some of its attributes. It will be recalled that this strain was derived from what might be termed a mixed culture and that it represents the small, fine colony component of that culture. On several occasions throughout the continued study of this culture it exhibited marked changes, not only with reference to its susceptibility to the bacteriophage, but in morphology as well. Because of the readiness with which this small colony derivative of the Wright strain grew in fluid media, during the early studies broth cultures were used throughout, without intervening transfers to solid media (fig. 4). After a series of such transfers, during which time the organisms had retained their susceptibility to bacteriophage, a gradually increasing resistance became apparent. This increased resistance was evidenced not so much by a developing refractoriness to lysis as by the fact that after an apparently complete lysis the period intervening before the development

of a secondary culture progressively diminished. At the end of about 15 serial transfers from broth to broth it became obvious that the culture had become transformed, although morphological study of the strain at this time failed to disclose profound differences as compared with the strain used in the initial tests. The tendency toward bipolar staining was possibly more in evidence and it seemed that that portion of the rod lying between the deeply-staining granules tended to take the stain less intensely. cursory examination of the culture in stained preparation at this time gave the impression of a streptococcus rather than of the diphtheria bacillus (fig. 5). This tendency progressed, but at no time did a culture ever mature without solid-staining rods, unmistakably diphtheria, being present with the cocci. Even when the single minute colonies have been fished from blood plates with the aid of the dissecting microscope an admixture of both types has always been encountered.

Reverting to the original Wright culture, a second isolation was made and the strain thus obtained was apparently identical with the first, stained preparations showing the small, bipolar staining rods, completely susceptible to lysis without subsequent secondary growth. Repeated serial passages with this derivative were effected and at the third passage evidence of secondary growth was apparent, and in successive passages this became more marked.

In studying this transformation it was noted that when secondary cultures followed lysis, platings from the control tubes invariably revealed colonies of the two types, comparable in all respects to the two types originally derived from the Wright strain. Platings of the secondary growth which appeared yielded colonies of but one type and these were identical with the colonies of the large, rough type as derived from the control tube.

It was obvious that the original Wright culture represented a strain tending toward "spontaneous" dissociation and this dissociative phenomenon was accelerated in the fluid medium. Examination of a serum-agar slant inoculated with the culture when the isolation was made and which showed only the almost invisible transparent growth typical of the substrain now revealed

many white, vigorously-growing colonies superimposed upon the original culture. These colonies and the bacteria composing them corresponded in every respect with the large, rough colonies. Fishings from the small, fine colonies of this culture, as well as from the original have been made periodically since this observation was made and they have been carried along through successive cultures simultaneously, both in broth and on agar.

The serial broth cultures do one of two things. They may become resistant to the bacteriophage, and when plated show, usually after a prolonged incubation, the presence of large, daughter colonies resembling in every particular the growth of the resistant strain, or they may exhibit a tendency toward spontaneous disappearance.

Filtrates made from such "suicide cultures" contain bacteriophage, and agar slants made from the culture when it first shows evidence of a failure to develop will show plaques. It may be stated again that serum-agar slants inoculated with the freshly isolated, susceptible derivative never show plaques or daughter colonies, and these cultures, when transferred to broth, grow, at first, very slowly so that incubation for 48 hours at least is necessary for obtaining growth from the initial transfers. These broth cultures are always more or less susceptible to bacteriophage. When a susceptible culture of this type is transferred to Besredka egg medium the morphological aspect of the organism becomes very distinctive. At the end of twenty-four hours smears reveal cocci only (fig. 6). These coccus forms are large, in diplo-arrangement, and seem characteristic of growth on this medium. That they represent a true phase of the development of this culture seems certain, since if smear preparations are made at short intervals during the period of growth upon this medium the transitional stages between the bacillary morphology and the diplococcus arrangement may be readily followed. These cocci are apparently associated with the accumulation of deeply-staining material at either end of the rod. The portion of the rod connecting these bipolar zones gradually loses its staining property and in some cases seems to disappear entirely.

In older cultures single cocci are occasionally found, but the

most common arrangement is in the diplococcus form with pairs of diplococci forming either tetrads or short chains. Cells of this coccus type are not to be confused with typical cocci, for at no time are these apparent cocci perfectly round in contour nor do they exhibit uniformity in size. They could not possibly be confused with staphylococci. When the cultures on the Besredka medium are allowed to stand for ten days or more before subculturing, the agar slant in almost every case will show at least half of the organisms to be typical diphtheria bacilli, belonging to the barred or solid-staining types (fig. 7). The colonies on plates are all alike and most of them contain both the coccoid and solid-staining forms. It must be admitted, however, that up to the present time a complete reversion from this coccus form to the original bacillary form has not been effected. Incidentally it may be added that broth cultures grown from such a culture on Besredka's medium resist bacteriophage.

The behavior of the susceptible Wright strain, that is, its tendency to give rise to the resistant type of colony or to "commit suicide," introduced the question as to whether the organism as we have studied it was not really a secondary culture. To study this problem filtrates from broth cultures which had been made some three weeks previously were prepared and 0.5 cc. of each filtrate was added to a tube of broth. Two drops of a broth culture of the susceptible diphtheria organism were added to each tube. Of the 36 filtrates thus tested 13 exerted no inhibitory action upon the test organism, another 13 showed a slight inhibitory effect, and the remaining 10 caused a complete inhibition of growth in the first passage. These cultures were again filtered and a second passage was made, using in each case one drop of the filtrate. In four instances no inhibition of growth followed, and in six but a slight clouding of the medium was obtained, while with 26 of the 36 strains lysis was complete, although readings made shortly after the inoculation showed that the susceptible strain underwent growth in all tubes. In the third passage with these culture-filtrates complete lysis was obtained in all except three and these exhibited such a slight turbidity that

they were read as partial lysis. All of the control tubes showed the absence of lysis and the controls containing known bacteriophage gave complete lysis. This third passage of the filtrates was prepared in duplicate and after the twenty-four-hour readings one set was returned to the incubator, and by the end of the fourth day secondary growth had developed in all except one. The duplicate set of tubes was held at room temperature and developed secondary growths in about a third of the tubes. In no instance, however, did the intensity of growth equal that obtained in the control tube.

While the tests above described were in progress, additional strains of diphtheria bacilli were being accumulated as rapidly as possible, the cultures being derived largely from field cultures supplied us by the Connecticut State Department of Health Laboratories and from clinical cases present in the New Haven Hospital. When an additional 40 such strains had been obtained they were tested in the same way and without exception the results were of the character above described. In no instance did a culture filtrate cause complete lysis after the first passage, although in about one-half of the cultures the filtrates caused more or less inhibition of growth. With the second passage, however, a complete absence of growth or lysis occurred in all but four cases, and these showed an inhibition. The twenty-four-hour reading of the tubes prepared from the third passage showed complete absence of growth in all tubes save the appropriate controls. At the end of four days about half of these tubes had yielded secondary cultures. Later a third set of ten cultures was prepared and these were filtered after they had grown in broth for three days. Nine of these failed to show evidence of lysis until the third passage. Upon the fourth passage all filtrates inhibited growth. Thus, while all of the cultures examined exhibited constancy in behavior as regards the ultimate result, there seemed to be differences of a quantitative nature indicating that if these field cultures really represent lysogenic strains, the readiness with which they yield demonstrable bacteriophage varies.

With the idea of determining whether this variability could be

referred merely to quantitative factors or whether quality also influences the result, ten cultures from among those previously examined were selected at random. Filtrates were prepared and these filtrates were subjected to ten passages with the susceptible diphtheria strain. After the tenth passage the filtrates were subjected to serial dilution for testing the relative potency of the bacteriophage races present. In 5 cases a complete bacteriophagy was obtained in a dilution of 1:500,000, in four it was complete at 1:3,500, and in one at 1:5,000. Obviously these races of bacteriophage are not of high potency, a fact which may be significant, but of even greater importance than this is the demonstration of the fact that such a difference actually exists. This indicates, of course, that the incitant for bacteriophagy was to be found in the filtrates derived from the diverse strains rather than in a splitting-out of bacteriophage from the susceptible strain used in all of the tests. It should, perhaps, be emphasized that this conclusion is merely indicated and is not proved by this experiment.

In addition to the large number of cultures subjected to examination as described above, another group of cultures was tested. These cultures were those described in a prior publication and were the strains which had been subjected to adverse environmental conditions and had yielded filter-passing organisms.

Cultures of the latter were made in broth and, after a period of incubation, filtrates were prepared and these were tested against the susceptible Wright strain. All of these filtrates contained lytic principles, but they were invariably of low potency, since, even after seven successive passages, nine of them failed to cause a complete and permanent lysis. That bacteriophage was present, however, could readily be seen from the second passage on throughout the series.

The nature of the Wright susceptible strain, fluctuating as it does, apparently on the basis of dissociative changes and selection, between resistance and susceptibility, inevitably introduces an element of uncertainty in matters of interpretation. Nevertheless, it appears certain that this strain possesses certain attributes which tend to set it apart from the other cultures which we have

studied. In the first place, it is the only culture from which a completely susceptible derivative has been recovered. Many of the other strains have exhibited a partial susceptibility, but a complete dissociation and segregation through colony types has not been attained. It is equally certain that this Wright culture, even in its susceptible form, is in reality, or through unknown causes may become, a lysogenic strain as is evidenced by its "suicide" behavior under certain circumstances. The facility with which it passes from the susceptible to the resistant state, with the accompanying morphological changes, appears to be peculiar to it, and while continued growth in broth accelerates the "susceptible-to-resistant" transformation, the true nature of the incitant is not known. While in the resistant form this culture is unquestionably lysogenic and must certainly carry the lytic component through its susceptible stage, it is equally true that many filtrates made of the culture during its sensitive periods have proved to be devoid of action on the organism itself. And yet, bacteriophage must have been present. This observation may very well serve as an example of the very insecure nature of the apparent stability of many cultures and to emphasize again the extreme subtlety of those forces competent to disturb equilibrium.

The intimate relationships between the bacteriophage and the elementary diphtheria organism are obscure, and the fact that all strains of diphtheria bacilli thus far isolated from field cultures yield lytic filtrates suggests that the diphtheria "bacillus" as we know it may be a most complex being. The coccoid forms occurring in the transformation of the susceptible derivative and the coccus stage appearing in the development of the bacillary form from the filter-passing stage may well be worthy of consideration. At the moment, however, it would seem that the most urgent problem is that associated with deriving additional strains susceptible to bacteriophagy. Until this is accomplished the studies here recorded must be regarded as but an isolated observation, and while suggestive, they do not justify the conclusion that the diphtheria bacillus as we know it is always a lysogenic culture, that is, a being in which the bacterium is

uniformly found in association with bacteriophage, possibly an obligate symbiosis.

As for diphtheria bacteriophage, it would seem to be fully as common as is bacteriophage for *B. coli* or for pyogenic cocci. Its demonstration in sewage, in patients, in convalescents, in field cultures, and under certain circumstances in dust and air, suggests that it may play a significant rôle under many circumstances, and offers to the epidemiologist an additional point of attack in the study of those conditions governing the behavior of communicable disease.

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A MARINE DENITRIFYING ORGANISM

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Received for publication, July 17, 1930

Bacterial denitrification has been known for over seventy years, and its deleterious action in reducing the available sources of combined nitrogen suitable for plant nutrition has been extensively studied. Much of our present information on the subject is derived from very early work, when the technique of bacteriology was still rudimentary, and when the nomenclature of microorganisms was in a confused state. Denitrifying organisms in particular were named without discrimination, so that it is scarcely possible to correlate the results of different workers. Organisms belonging to the same species have often been described under different names, and different species have undoubtedly been classed under the same specific name. This is particularly well illustrated by the term *Bacillus denitrificans*, which has been applied successively to many different species whose sole similarity is their ability to denitrify. This confusion of identity probably accounts for the apparently discrepant results and lack of agreement of early workers.

The writer, in planning more detailed work on denitrification, selected one of the several unknown denitrifying organisms isolated from the Clyde Sea Area. In view of the experimental work already completed (Lloyd and Cranston, 1930; Cranston and Lloyd, 1930), a description of the organism is now given. Cultures have been deposited with the American Collection of Type Cultures, and with the British Collection of Type Cultures, Lister Institute, London.

This strain, known as Laboratory Organism XIV, was isolated from sea water in Loch Striven off the west coast of Scotland at

a depth of 20 fathoms. Similar strains were frequently isolated from this and other places in the Clyde Sea Area. The following are the principal diagnostic features of the organism:

I. MORPHOLOGY

The cells are usually single small slender vibrio or straight forms about 1.5μ in length and 0.4μ in breadth. Organisms in a newly inoculated culture or in a senescent one are non-motile, but during the phase of rapid growth, the majority of cells are motile. Cilia have not been seen, possibly on account of the small size of the bacillus. It is Gram-negative.

Spores were not observed directly, but stained preparations sometimes showed darkly coloured areas. However, an eight months culture was found to be viable after boiling for ten minutes, whereas a culture fourteen hours old was killed by five minutes' heating to 68°C . Thus the cells pass without demonstrable change into a resistant arthrospore state.

II. CULTURAL CHARACTERS

Agar slope. After eighteen hours at 37°C . a thin tenacious wrinkled film appears, closely adherent to the surface of the medium. The margin appears to the naked eye entire, but is minutely folded; when the surface of the agar is moist, a thin bluish structureless zone surrounds the main region of growth. The surface wrinkles give a finely reticulate appearance; near the edge these wrinkles are parallel, so that at a magnification of ten diameters the margin appears frilled.

Agar colonies. Sub-surface colonies are small and structureless, brownish-white, rounded or lenticular, growing to a diameter of 2 mm. in seven days at room temperature. Surface colonies are roughly circular, with a frilled edge, a slight elevation, and a variously wrinkled surface. The sculpturing of the colony depends in part upon the consistency of the medium, and in part upon the age of the parent culture. When an agar plate is prepared from a parent culture which is young, i.e., about fifteen hours at 37°C . or twenty-four hours at 32°C . or thirty-six hours at room temperature, the resulting colonies are thin, translucent,

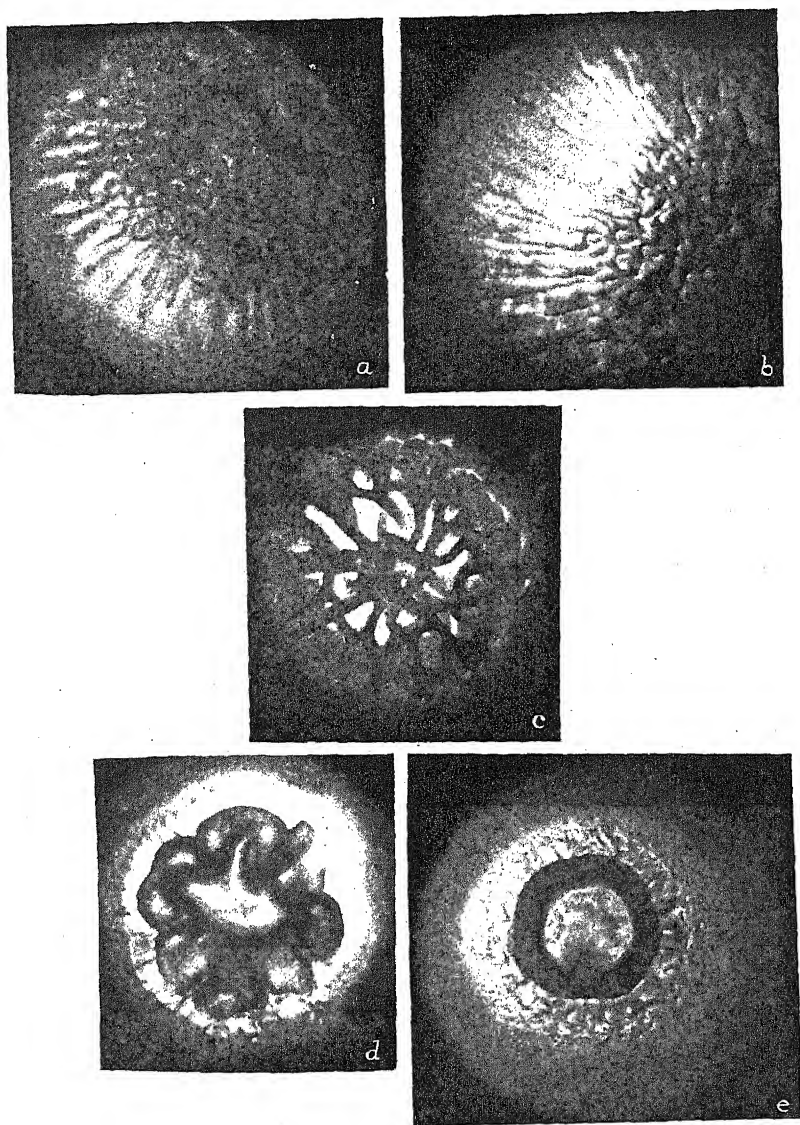


FIG. 1. DIFFERENT TYPES OF MATURE SURFACE COLONIES ON AGAR

a, edge of colony sub-cultured from three-day parent culture; *b*, edge of colony from three-day parent culture; *c*, entire colony, typical form with radiating costae and structureless peripheral zone; from seventeen-day parent culture; *d*, sub-cultured from twenty-hour culture; *e*, sub-cultured from twenty-hour culture. Magnification sixteen diameters.

marked with radiating or reticulate costae, and only very slightly raised above the surface of the medium. When, on the other

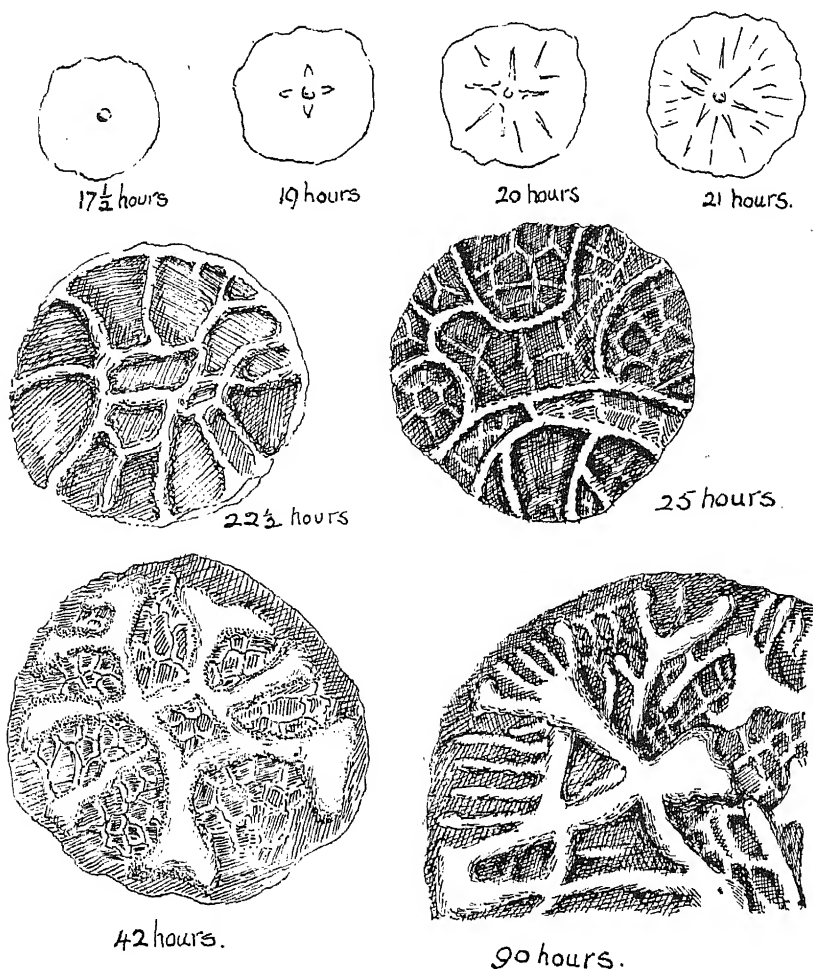


FIG. 2. STAGES IN THE DEVELOPMENT OF COSTAE IN A SINGLE SURFACE AGAR COLONY INCUBATED AT ROOM TEMPERATURE
Magnification thirty-three diameters

hand, a plate culture is made from an old parent culture, the resulting colonies are cream-brown and elevated into coarse,

opaque and irregularly disposed wrinkles, often with a clear structureless zone outside. Figure 1 shows different types of mature colonies. The macroscopic difference is so striking that such colonies might erroneously be assigned to different genera. Figure 2 shows successive stages in the development of costae in one colony.

Gelatin cultures. Gelatin stab cultures show a line of discrete colonies without liquefaction. Plate cultures resemble morphologically those on agar.

Broth cultures. Growth in broth is turbid, with a fine white sediment and a tenacious wrinkled brownish surface film. The nature of the film is determined by the temperature of incubation; at 37°C. a structureless opalescent film is produced, but at lower temperatures the film becomes progressively thicker, more horny and more wrinkled.

This film formation is apparently due to a concentration of the organisms at the surface of the broth where oxygen is more abundant, for when oxygen is available from some constituent added to the broth, as for example from nitrate, the film is not formed until all the nitrate is used.

Potato slope. No growth.

Loeffler's blood serum. Growth poor, with no liquefaction.

III. BIOCHEMICAL REACTIONS

Purple milk. Slight alkalinity occurs; there is no peptonisation and no curd.

Sugar reactions. Acid, but no gas, is formed slowly in 14 days at room temperature from glucose and levulose; there is no fermentation with mannitol, lactose, dulcitol, galactose, sucrose or maltose.

Chromogenesis. No marked colour is produced on any medium; the colour of the colonies ranges from slightly opalescent white young colonies to brownish or, less commonly, yellowish older colonies.

Decomposition products of peptone. Indol, ammonia, hydrogen sulphide, and nitrite are not formed in peptone water.

Decomposition of nitrates. Nitrates are reduced very readily

under both aerobic and anaerobic conditions to nitrites and then to free nitrogen, but not to ammonia. Under suitable conditions any nitrite or nitrate present in the culture medium is reduced completely to nitrogen. (Cranston and Lloyd, 1930). A brief summary of the experimental results obtained with this organism is given below.

Other reductions. Methylene blue, indigo, selenates, and tellurates are reduced, but not sulphates or chlorates.

IV. PHYSICAL REQUIREMENTS

Optimum pH, 8.2.

Optimum temperature, 37°C. It is interesting that the optimum temperature for this organism is so much higher than that of the habitat from which it was isolated.

V. DESIGNATION

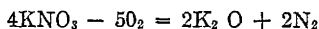
The name *B. costatus* is proposed for the organism, as it is descriptive of its characteristic colony formation.

VI. SUMMARY OF RESULTS OBTAINED WITH THE ABOVE ORGANISM

The course of denitrification in bacterial cultures has been studied, using the apparatus devised by Cranston (1930). By measuring the amount of gas produced during given intervals of time, a *denitrification curve* was obtained, and from this a *rate of denitrification curve* calculated for any culture. These curves were found to resemble certain growth curves. A denitrification curve shows the amount of nitrogen formed as waste metabolite, and hence indicates the aggregate number of bacteria that have lived in the culture; a rate of denitrification curve, on the other hand, may be correlated with a growth curve showing the number of bacteria present at any given time. However, in interpreting the growth of a denitrifying culture in terms of nitrogen evolved, it was noted that the relationship is not a simple one, and that the number of viable organisms in a culture is not necessarily directly proportional to the rate of gas evolution at any given time.

The process of denitrification is one of reduction, and its phys-

iological utility to the organism is the liberation of oxygen which may be used for respiration either instead of, or as supplement to, free atmospheric oxygen. The organism described has evidently obtained its nitrogen as amino-nitrogen, since the nitrogen of the nitrate supplied was not utilised. Korsakova (1927) found that denitrification took place in stages, nitrates being reduced to nitrites, then to an intermediate compound, probably hyponitrite, and finally to nitrogen. Cranston and Lloyd (1930) have shown that in the complete equation for denitrification:



2.5 molecules of oxygen are consumed for each molecule of nitrogen evolved, but the amount of oxygen liberated at each stage is different:

- (i) *Nitrate to nitrite.* $2\text{KNO}_3 - \text{O}_2 = 2\text{KNO}_2$
- (ii) *Nitrite to hyponitrite.* $2\text{KNO}_2 - \text{O}_2 = \text{K}_2\text{N}_2\text{O}_2$
- (iii) *Hyponitrite to nitrogen.* $2\text{K}_2\text{N}_2\text{O}_2 - \text{O}_2 = 2\text{K}_2\text{O} + 2\text{N}_2$

Thus, if the second stage is completed before the third is begun, four-fifths of the available oxygen would be liberated before any nitrogen is evolved at all. Where a culture has sufficient nitrate to provide the requisite oxygen by stage (i), then no nitrogen may be liberated, although the culture may show a healthy growth. Hence the production of the waste metabolite nitrogen is not a sure guide to the bacterial population in a culture.

Conditions affecting denitrification. a. *Initial concentration of nitrate.* From the foregoing it is seen that the initial concentration of nitrate or nitrite in the culture is an important factor, and one whose significance has not hitherto been realised. Thus, when sufficient oxygen is obtained for the respiratory needs of the culture by reduction stages (i) and (ii), there is no denitrification, using the term in the strict sense of nitrogen-liberation.

b. *Temperature.* As the temperature departs from the optimum in either direction, the rate of denitrification becomes progressively slower. A temperature of 45°C., though it does not destroy the organism, inhibits growth and therefore denitrification.

c. *Hydrogen ion concentration.* When the medium is more acid

than the optimum, the rate of growth is diminished, although the aggregate number of bacteria as measured by the amount of nitrogen evolved is unaffected.

SUMMARY

A description is given of a denitrifying organism isolated from sea water off the Scottish coast, and a brief account is also given of the experimental results on denitrification already obtained with this organism.

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SAPROPHYTIC AND SECONDARY MICROÖRGANISMS OCCURRING IN THE RESPIRATORY TRACTS OF DOMESTIC FOWLS AND CHICKENS IN HEALTH AND IN DISEASE¹

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Received for publication, August 7, 1930

This paper is based primarily upon a microbiological study of the larynx, trachea, syrinx, bronchi, and lungs of 56 fowls one year or more of age and 14 chickens about six weeks old. Streaks, from the various portions of the respiratory tract studied, were made on chicken infusion agar, with and without fowl blood, and incubated at 37°C. from twenty-four to forty-eight hours, or until sufficient growth appeared for colony fishing and isolation of bacteria. Most of the microörganisms isolated and studied grew best upon chicken infusion agar. In fact only two organisms, both of which proved to be streptococci, were hemophylic. A spirochete, which, so far as the author is aware, has not been reported in the United States before, failed to grow upon any of the culture media used in this study. However, direct smears were made on slides, stained, and studied under the microscope.

The birds were divided into the following groups after careful clinical, postmortem, and microbiological examinations had been made:

Group I. Infectious trachitis ²	42
Group II. Pullorum disease.....	6
Group III. Chronic laryngitis.....	7
Group IV. Avian paralysis.....	5
Group V. Normal healthy controls.....	10

¹ Massachusetts Agricultural Experiment Station Contribution No. 113.

² Other names that have been used for this disease are infectious bronchitis, laryngo-trachitis, chicken flu, and Canada flu.

The microörganisms isolated from these birds in cultures and in smears fell into groups as follows:

Staphylococcus.....	40
Spirocheta.....	33
Escherichia.....	20
Micrococcus.....	8
Streptococcus.....	5
Eberthella.....	5
Alcaligenes.....	3
Bacillus.....	2
Pseudomonas.....	2
Sarcina.....	2
Pasteurella.....	1
Torula.....	1
Saccharomyces.....	1

INFECTIOUS TRACHITIS

According to the preceding general classification, the following microörganisms were isolated from the 42 birds dying of infectious trachitis:

Staphylococcus.....	31
Spirocheta.....	21
Escherichia.....	14
Micrococcus.....	6
Streptococcus.....	5
Pseudomonas.....	2
Alcaligenes.....	2
Eberthella.....	2
Bacillus.....	2
Pasteurella.....	1
Torula.....	1
Saccharomyces.....	1

On the basis of sub-cultural, morphological, pathological, and chromogenic characteristics, the staphylococci isolated from the birds dying of infectious trachitis were further classified as follows:

<i>Staphylococcus albus</i> , Rosenbach.....	22
<i>Staphylococcus aureus</i> , Rosenbach.....	4
<i>Staphylococcus epidermidis</i> , (Welch) Bergey <i>et al.</i>	4
<i>Staphylococcus citreus</i> , Migula.....	1

Staphylococcus albus was isolated from 22 of the 42 cases of infectious trachitis, yet in most of the instances studied this

organism appeared to be a harmless parasite in the respiratory tract. The most that can be said of it from a pathogenic point of view is that it may have played the rôle of a secondary invader in acute respiratory diseases of fowls and chickens. In one fowl it was isolated in pure culture from an abscess which had destroyed the right lung, and in six other cases it was present in congestion, inflammation, and pyemia of the lungs of chickens dying of infectious trachitis, complicated with clinical and pathological symptoms of broncho-pneumonia.

Staphylococcus aureus, *citreus* and *epidermidis* appeared as harmless parasites in the larynx, trachea, and bronchial tubes of nine of the birds studied. In two cases *Staphylococcus albus* and *Staphylococcus aureus* were isolated in mixed cultures, but for the most part pure colonies were fished from the plates on which the streaks were made.

Spirochetes were found in 21 of the 42 cases of infectious trachitis examined. This was by far the most interesting organism discovered in this study because it was new to the writer. The symptoms of infectious trachitis of fowls resembled broncho-spirochetosis of man in the Orient so much that the writer was led to believe for a while that he had discovered the causative agent of infectious trachitis. This organism is a protozoan, closely related to *Spirocheta buccalis* of higher animals. It was found in the secretions of the beak, throat, pharynx, larynx and trachea of some of the fowls examined. The author first observed it in the trachea of fowls dying of infectious trachitis. The spirochete may have been of some importance as a secondary invader, although it did not penetrate deeper than the epithelium, as the microörganism could not be found in the mucosa, submucosa, or musculature of the larynx, trachea, and bronchi, when stained by Levaditi's technique and examined histologically.

The spirochete averaged 2.5μ long and 0.1μ wide. The dimensions of the largest spirochete found were 5μ long and 1.2μ wide. The smallest was 1.5μ long and $.08\mu$ wide. It was best stained by 2 per cent gentian violet. The smears were air-dried as the microörganism was easily destroyed by heat. It was found that India ink and Romanowsky stains could be used for the demon-

stration of the organism on slides. Two, three, and sometimes four objects resembling vacuoles appeared in smears stained by gentian violet after standing for a month or longer. The spirochetes in old smears degenerated, even when kept in the dark, leaving only faint spiral outlines on the slides.

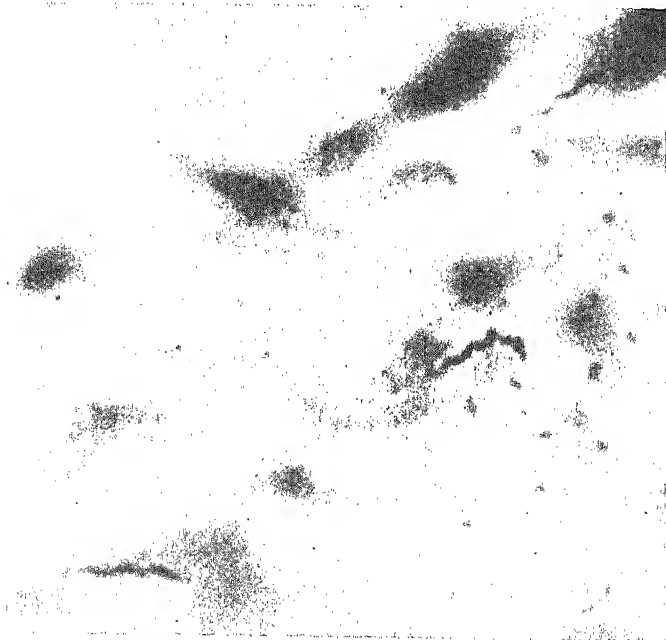


FIG. 1. MICROPHOTOGRAPH OF SPIROCHETES FROM TRACHEA OF DOMESTIC FOWL.
DIRECT SMEAR STAINED BY 2 PER CENT GENTIAN VIOLET. MAGNIFIED 1900
DIAMETERS.

This spirochete was not primarily pathogenic to fowls and chickens, although it may have been involved in secondary and chronic infections of the respiratory tract, as indicated above. So far as our studies are concerned, this microorganism bears no relation to *Spirocheta gallinarum* which is a blood parasite. The spirochete under discussion was not found in the blood of any of the cases studied. Morphologically, *Spirocheta gallinarum* is a slender parasite with wavy spirals, while the organism under

consideration is comparatively thick and the spirals are acute. The spirochete was found in about half of the adult birds examined, but the other half became carriers after swabbing the larynx and trachea with contaminated secretions. The inoculated birds showed no serious effects from the treatment, although some of them developed chronic laryngitis.

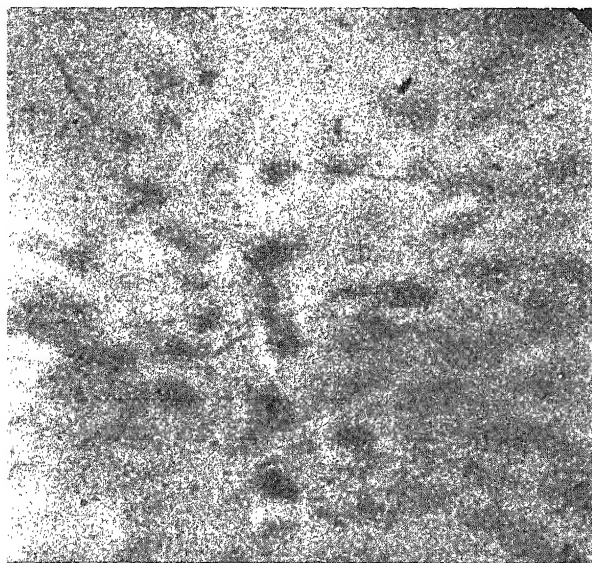


FIG. 2. MICROPHOTOGRAPH OF SPIROCHETES FROM TRACHEA OF DOMESTIC FOWL. SAME FIELD AS SHOWN IN FIGURE 1. MAGNIFIED 950 DIAMETERS.

The spirochetes are stained by 2 per cent gentian violet.

Since the completion of this study, the writer's attention has been called to the fact that tracheal spirochetes were discovered in fowls in Germany (1927). The German species differ somewhat from those described herein. One of the spirochetes described by the German investigators was long, slender, and irregular in regard to spirals, while the other species resembled the one reported in this paper in every respect, except that whip-like flagella were carried at either one or both ends. Flagella were not demonstrated in the spirochetes studied in this investigation.

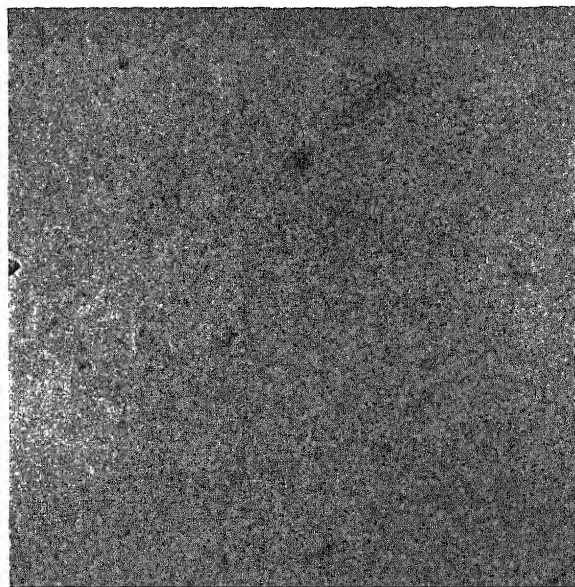


FIG. 3. MICROPHOTOGRAPH OF SPIROCHETES FROM TRACHEA OF DOMESTIC FOWL, STAINED BY 2 PER CENT GENTIAN VIOLET, AND MAGNIFIED 1900 DIAMETERS.

The spirochete in the center has degenerated, leaving a halo of its former outline.

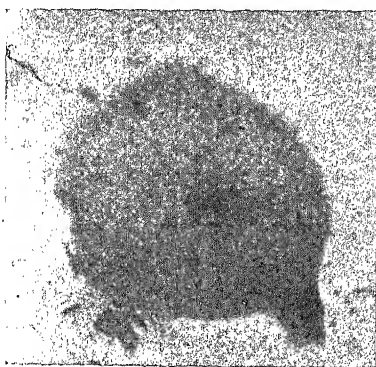


FIG. 4. MICROPHOTOGRAPH OF EPITHELIAL CELL FROM TRACHEA OF DOMESTIC FOWL SHOWING SPIROCHETES WITHIN. MAGNIFIED 1900 DIAMETERS. GENTIAN VIOLET STAIN.

Escherichia were found in 12 of the fowls and 2 of the chickens dying from infectious trachitis. These organisms were classified as follows, according to Bergey's *Manual of Determinative Bacteriology*:

<i>Escherichia communior</i> (Durham) Bergey <i>et al.</i>	6
<i>Escherichia coli</i> (Escherich) Castellani and Chambers.....	5
<i>Escherichia gastrica</i> (Ford) Bergey <i>et al.</i>	1
<i>Escherichia alkalescens</i> (Ford) Bergey <i>et al.</i>	1
<i>Escherichia pseudocoloides</i> , Castellani and Chalmers.....	1

All of these microörganisms normally occur in the alimentary canal of domestic fowls. It is quite likely that many of them found their way into the respiratory tract from the alimentary canal after inflammation had set in and the ciliated epithelium had been destroyed. While these microörganisms did not appear to be directly involved in the inflammatory processes taking place in infectious trachitis, yet they were often found in great numbers in the secretions of the trachea during the febrile stages of the disease.

Six cultures of micrococci were isolated from four fowls and one chicken dead of infectious trachitis. These cultures fell into two unidentified groups, as follows:

Group I consisted of four cultures of Gram-negative spheres occurring in pairs. The colonies on chicken infusion agar were isolated, small, circular, grayish white, glistening, and slightly raised in the centers. Gelatin was not liquefied. A turbid opalescent growth appeared in plain broth after twenty-four hours' incubation at 37°C. Acid was slowly formed in litmus milk. The organism curdled milk after three days' growth. Acid was produced in glucose, lactose, sucrose, maltose, and mannitol. The micrococci were aerobic and non-pathogenic for fowls and chickens.

Group II consisted of two cultures. The organisms in this group resembled those in Group I, except that both acid and gas were formed in glucose, lactose, maltose, and mannitol. Also, sucrose and dulcitol were made alkaline at first, later turning acid and producing gas.

Five cultures of streptococci were isolated in this study from

fowls dying of infectious trachitis. These cultures also fell into two groups when classified according to morphological, physiological, and pathological characteristics. The first group, consisting of three cultures, produced long chains and was neither hemophilic nor hemolytic. The other group, consisting of two cultures, grew best on chicken infusion blood agar and was decidedly hemolytic. While these microorganisms were not serologically typed, they were divided into two groups as stated above. The first group of microorganisms was not named, because it did not correspond to any of the types given in Bergey's *Manual of Determinative Bacteriology* for the classification of streptococci, but the characteristics of the second group were like those of *Streptococcus pyogenes* Rosenbach. So far as could be determined by these experiments, the three cultures classified as non-hemolytic streptococci were harmless saprophytes living on the mucous membrane of the respiratory tract of the domestic fowls studied. But the hemolytic strains may have been involved in the hemorrhagic conditions existing in the larynx and trachea of the fowls from which they were isolated.

Two trachitis cases of long standing yielded pure cultures of *Pseudomonas aeruginosa* (Schröter) Migula from green pus forming pockets in the larynx just below the rima glottis. In these cases *Pseudomonas aeruginosa* was quite likely a secondary invader, as the swabbing of the trachea of healthy birds did not lead to inflammation or disease.

In this study two cultures resembling *Alcaligenes bronchisepticus* (Ferry) Bergey *et al.*, freshly isolated from the trachea of birds dying of infectious trachitis, did not prove to be pathogenic to healthy fowls and chickens when inoculated intratracheally. The identity of these microorganisms was not definitely established by animal inoculation. They grew abundantly on chicken infusion agar under aerobic conditions, which differentiates them quite readily from *Alcaligenes abortus* (Bang) Bergey *et al.*, which has been reported as isolated from the domestic fowl (1929).

A culture resembling *Pasteurella avicida* (Kitt) Trevisan, morphologically and subculturally, was isolated from the trachea of one of the birds dying of infectious trachitis. This culture was

not pathogenic for hens and chickens when inoculated by tracheal swab or hypodermically, or when fed in the drinking water. However, this may have been a culture of the fowl cholera organism which had so lost its virulence that several continuous passages through birds would have been necessary in order to restore its pathogenicity. Since the writer was interested only in the possible relation of these organisms to infectious trachitis, the animal passages were not continued beyond the third transfer.

Two cultures, one from a fowl and another from a chicken dying of infectious trachitis, were classified as *Eberthella septicemia* (Riemer) Bergey *et al.*, although the biochemical reactions did not correspond exactly with the descriptions given for this micro-organism in Bergey's *Manual of Determinative Bacteriology*. According to Bergey's *Manual*, *Eberthella septicemia* produces acid in glucose only. The organism described herein, as *Eberthella septicemia*, formed acid in both glucose and maltose. However, the cultures were not pathogenic to either fowls or chickens. So far as could be determined, these organisms were not involved in the inflammatory conditions existing in the disease under investigation.

The two spore-forming cultures isolated from the trachea of fowls dying from infectious trachitis belonged to the subtilis group. They occurred either as harmless saprophytes in the birds studied or as contaminations in the culture media used in these experiments.

A torula and a yeast culture were isolated from the trachea of two of the fowls dying from infectious trachitis. The torula culture produced acid in glucose, lactose, sucrose, and maltose. The yeast or saccharomyces culture formed acid and gas in glucose and maltose and slight acid in sucrose. These micro-organisms were not pathogenic for fowls. It is quite likely that they got into the trachea from the crop, via esophagus, pharynx, and larynx during a spasm of coughing, vomiting, and gasping for breath during the dyspneic stage of infectious trachitis.

PULLORUM DISEASE

The microbiological flora of the respiratory tract of six fowls having chronic focal infections of pullorum disease were included

in these experiments for comparative study. The following cultures were isolated from these fowls at autopsy:

<i>Escherichia communior</i> (Durham) Bergey <i>et al</i>	3
<i>Escherichia coli</i> (Escherich) Castellani and Chalmers.....	2
<i>Sarcina</i> , unidentified species	1

All of these cultures, except the *sarcina*, were the same as those found in birds having infectious trachitis and need no further discussion here. However, it is pertinent to point out that *Salmonella pullora* was not isolated from the respiratory tracts of the birds studied in this investigation. The *sarcina* was not pathogenic to domestic fowls and chickens.

CHRONIC LARYNGITIS

Seven cases of chronic laryngitis were studied. The spirochete described above was found in the secretions of the larynx of all of these birds. Cultures of *Staphylococcus albus* were isolated from four of the fowls, while two others had contagious epithelioma. These microorganisms may have been responsible for the chronic condition existing in the larynx, although it is doubtful if they had anything directly to do with the chicken-pox.

AVIAN PARALYSIS

Five of the birds selected for this study were suffering from avian paralysis. The following cultures and smears were studied from this group:

<i>Spirocheta</i>	2
<i>Eberthella bentolensis</i> , Castellani and Chalmers.....	2
<i>Staphylococcus albus</i> , Rosenbach.....	1

The spirochete and the culture of *Staphylococcus albus* were the same as those previously described in this paper and need no further discussion. However, the species of *Eberthella* differed from the microorganisms found in any of the other birds. Moreover, it is not likely that *Eberthella bentolensis* had anything to do with the paralysis of these birds, as it appeared to be innocuous to healthy fowls and chickens over a period of six months.

NORMAL HEALTHY CONTROLS

Ten fowls were kept as healthy controls. Seven of these birds were found to be carriers of microörganisms in their respiratory tracts. The remaining three seemed to be free from such parasites as would grow on the culture media or stain with the dyes in direct smears used in these experiments. The following cultures and smears were secured from the control birds:

<i>Staphylococcus albus</i> , Rosenbach.....	4
<i>Spirocheta</i>	3
<i>Micrococcus</i> , unidentified species.....	2
<i>Escherichia communior</i> (Durham) Bergey <i>et al.</i>	1
<i>Alcaligenes bronchisepticus</i> (Ferry) Bergey <i>et al.</i>	1
<i>Eberthella septicemia</i> , Bergey <i>et al.</i>	1
<i>Sarcina lutea</i> , Schröter.....	1

The *Staphylococcus*, *Micrococcus*, *Escherichia*, *Alcaligenes*, and *Eberthella* cultures were the same morphologically and sub-culturally as have been described already in this paper under infectious trachitis. *Sarcina lutea* did not occur in any of the diseased birds included in this investigation. It was found in only one of the controls. There was nothing to indicate that this microörganism was in any way pathogenic to fowls.

SUMMARY

1. Microbiological cultures and smears were made from the larynx, trachea, syrinx, bronchi, and lungs of 56 domestic fowls and 14 chickens. Forty-two of the birds had died of infectious trachitis, six had pullorum infection, seven were suffering from chronic laryngitis, five were affected with avian paralysis, and ten were kept as healthy controls.

2. *Staphylococci* were isolated from the respiratory tracts of 73.81 per cent of the birds having trachitis, 57.14 per cent of those suffering from chronic laryngitis, and 40.00 per cent of the healthy controls.

3. *Spirochetes* were found in 50 per cent of the birds dying of infectious trachitis, 100 per cent of the birds having chronic laryngitis, 40 per cent of the fowls suffering from avian paralysis, and 30 per cent of the healthy fowls and chickens.

4. *Escherichia* of intestinal origin were found in the respiratory tracts of 33.33 per cent of the birds having infectious trachitis, and 83.33 per cent of the pullorum infected fowls. Only 10 per cent of the normal controls were found harboring *Escherichia* in the respiratory tract.

5. *Alcaligenes* cultures were isolated from 4.76 per cent of the trachea of fowls affected with infectious trachitis and 10 per cent of the control birds.

6. Two cultures of hemolytic streptococci were isolated from the hemorrhagic inflammation of the larynx and trachea of two types of so-called bloody infectious trachitis in fowls.

7. Two cultures of *Pseudomonas aeruginosa* were isolated from pus pockets in the larynx of two birds having prolonged cases of infectious trachitis.

8. A culture resembling *Pasteurella avicida* morphologically and sub-culturally was isolated from the trachea of one fowl dying from infectious trachitis. The culture might have lost its virulence for it did not produce fowl cholera in either fowls or chickens.

9. In addition to the above, *Micrococcus*, *Bacillus*, *Eberthella*, *Sarcina*, and one culture each of *Torula* and *Saccharomyces* were isolated from the respiratory tracts of the birds studied in this investigation. These cultures were not pathogenic to any of the fowls and chickens tested.

CONCLUSION

The microbiological flora of the respiratory tract of domestic fowls and chickens is varied in health and in disease. The number and variety of microorganisms are increased in acute respiratory diseases. This increase in microbial flora may be due to the destruction of the ciliated epithelium of the mucous membrane and the increase in secretions and other inflammatory products which are utilized as food by the invader.

While this investigation did not produce sufficient etiological evidence to implicate any of the microorganisms isolated and studied herein as the primary cause of infectious trachitis, nevertheless, pathological indications demonstrated *Staphylococcus albus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and a spirochete, n. sp., as important secondary invaders.

ACKNOWLEDGMENT

The microphotographs of the spirochetes described in this article are due to the kindly assistance of Dr. William H. Davis of the Department of Botany, Massachusetts Agricultural College.

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THE EFFECT OF TEMPERATURE UPON THE PRODUCTION OF HYDROGEN SULPHIDE BY *SALMONELLA PULLORUM*¹

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Received for publication August 8, 1930

A general study of hydrogen sulphide production by *Salmonella pullorum* was begun in 1927 because of the irregular results reported by Mallmann (1925) as contrasted to the positive findings of Hadley *et al.* (1918), Mulsow (1925), Spray and Doyle (1921), May and Tibbetts (1923), May and Goodner (1926), Bushnell *et al.* (1926) and others.

Strikingly different results were soon noted by two workers who used the same strains, lead acetate agar and general technique. However, upon investigation it was found that two decidedly different incubation temperatures were being used even though incubations were made in the same laboratory incubator. The temperatures were 34° and 43°C. on top and bottom shelves respectively. The electric heating units were on the bottom. These results, therefore, led to the study reported here.

TECHNIQUE

Three hundred strains of *Salmonella pullorum* were used. They came from widely separated sources and had widely different dates of isolation; 250 were isolated by the writer over a period of five years and 50 were secured from eight Agricultural Experiment Stations. Of the total number 255 were isolated from chicks, 15 from septicemic infections in adult fowls, 18 from the

¹ Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station as Technical Paper No. 511.

TABLE 1
Relative amounts of hydrogen sulphide produced at various temperatures

STRAIN	TEMPERATURE OF INCUBATION											
	20°C.			30°C.			37°C.			44°C.		
	Period of incubation in days											
	1	2	4	1	2	4	1	2	4	1	2	4
1	S	2	4	1	4	4	tr.	2	2	tr.	S	S
2	S	2	4	S	4	4	S	1	2	—	—	tr.
3	S	1	3	2	4	4	1	3	3	—	—	—
4	S	1	4	1	2	4	1	3	3	—	—	—
5	—	—	—	—	—	tr.	—	—	—	—	—	—
6	S	2	4	2	4	4	tr.	1	3	—	—	tr.
9	—	—	—	—	tr.	tr.	—	—	—	—	—	—
12	—	—	—	—	—	—	—	—	—	—	—	—
13	—	—	S	—	tr.	tr.	—	—	—	—	—	—
14	1	3	4	2	4	4	2	4	4	—	—	—
15	S	1	3	1	4	4	1	3	4	tr.	S	1
16	S	3	3	2	4	4	S	2	2	—	—	—
17	1	2	4	3	4	4	1	3	4	—	—	tr.
21	—	—	—	tr.	tr.	3	tr.	tr.	1	—	tr.	tr.
24	S	2	4	3	4	4	2	4	4	tr.	tr.	tr.
25	S	1	4	2	4	4	2	3	3	—	—	—
26	1	2	4	2	4	4	S	1	2	—	—	—
27	—	—	tr.	S	S	1	—	tr.	S	—	—	—
29	1	3	4	1	4	4	S	1	3	—	—	—
30	S	1	2	1	3	4	S	2	2	—	—	—
31	S	S	2	1	4	4	S	2	3	tr.	tr.	tr.
32	S	S	3	—	2	4	tr.	S	2	—	—	—
33	S	1	3	tr.	1	3	tr.	2	3	—	tr.	tr.
36	1	3	4	3	3	4	1	2	4	—	—	—
38	S	1	3	1	3	4	1	2	3	—	—	—
39	—	—	—	—	—	—	—	—	—	—	—	—
40	S	1	3	S	1	2	—	tr.	1	—	—	—
41	1	2	4	1	4	4	S	1	2	tr.	tr.	1
42	S	3	4	2	4	4	1	2	3	—	—	—
43	2	4	4	2	3	4	1	3	4	tr.	tr.	tr.
44	S	1	2	S	S	1	S	1	2	tr.	tr.	tr.
45	—	S	2	S	1	3	tr.	S	2	—	—	—
48	1	2	3	3	4	4	1	3	4	—	—	—
49	S	1	4	1	2	4	S	1	3	—	—	tr.
50	1	1	3	2	3	4	2	4	4	—	—	—
51	1	2	4	1	3	4	2	3	4	tr.	tr.	S

The amounts of H_2S are recorded in six degrees—4 meaning complete blackening and 3, 2, 1, S (slight) and tr. (trace) indicating decreasing amounts.

TABLE 1—*Concluded*

STRAIN	TEMPERATURE OF INCUBATION											
	20°C.			30°C.			37°C.			44°C.		
	Period of incubation in days											
	1	2	4	1	2	4	1	2	4	1	2	4
54	1	2	4	2	4	4	1	2	3	—	—	—
55	1	2	4	2	4	4	1	2	4	S	S	2
58	1	3	4	2	3	4	tr.	tr.	tr.	—	—	—
60	S	2	4	2	4	4	1	4	4	—	—	—
61	S	1	4	1	3	4	—	—	tr.	—	—	tr.
62	S	1	3	S	2	4	1	3	3	1	2	2
64	1	4	4	3	4	4	S	1	2	—	—	—
65	S	2	4	2	3	3	1	2	3	—	—	—
66	1	3	4	2	4	4	S	2	2	—	tr.	tr.
67	—	tr.	tr.	—	—	—	—	—	—	—	—	—
68	1	3	4	3	4	4	3	4	4	—	tr.	tr.
69	1	3	4	2	3	4	—	tr.	S	tr.	tr.	tr.
70	S	2	4	S	4	4	—	—	tr.	—	—	—
71	1	3	4	3	4	4	1	2	3	tr.	S	S

ovaries of carriers, and 12 from eggs. Both the aerogenic and anaerogenic types were represented.

Difco dehydrated lead acetate agar was used throughout this study. It was prepared with distilled water, tubed in about 10 cc. amounts, autoclaved at 15 pounds pressure for twenty minutes and allowed to solidify in an upright position. Inoculations were made by thrusting the needle between the glass and the agar. Sufficient tubes were inoculated at one time to furnish one for each temperature being studied. A blackening along the line of inoculation was considered indicative of H_2S production.

Incubations were made in electric incubators adjusted to 20°, 25°, 30°, 34°, 37°, 40°, 42° and 44°C. Readings were made after one, two, four and seven days. Longer incubation periods were used in the first part of the study but were discontinued because there was practically no change after four days.

EXPERIMENTAL

Fifty strains were tested in 1927. The results presented in table 1 show practically no H_2S production at 44°C., considerable

inhibition at 37°C., maximum production at 30°C., with marked variations between strains and either very little or no H₂S produced by six strains. The negative strains were all anaerogenic. This point will be discussed later.

TABLE 2
Representative average results after four days incubation

STRAIN	TEMPERATURE OF INCUBATION					
	25°C.	30°C.	34°C.	37°C.	40°C.	42°C.
1	4	4	3	2	S	S
6	4	4	4	3	S	tr.
26	4	4	3	2	1	—
41	4	4	3	2	1	1
65	4	4	4	3	1	—
106	3	3	2	1	tr.	—
165	3	3	3	1	—	—
190	4	4	4	4	2	tr.
210	4	4	3	2	tr.	—
223	1	2	2	1	—	—
240	4	4	3	2	tr.	—
260	4	4	4	2	1	tr.
280	2	2	1	tr.	—	—
300	4	4	4	4	2	tr.
304	2	3	3	2	1	—
313	1	2	2	1	—	—
400	4	4	3	3	1	tr.
409	3	4	4	3	1	—
420	4	4	4	2	tr.	—
455	4	4	4	3	2	tr.
12	—	—	—	—	—	—
77	—	—	—	—	—	—
Average..	3.0	3.3	2.8	1.9	0.7	0.1

From 1928 to 1930 these strains and 250 additional ones were studied at various temperatures between 25° and 42°C. with especial attention given to a comparison of 30° to 34°C. with 37°C.

Since the accumulated data are too voluminous to give in detail, it seems necessary to present them summarized in a brief discussion. In general the results confirmed those in table 1

although the percentage of negatives was considerably less. Representative results given in table 2 show that, in general, inhibition begins at about 34°C. and gradually increases until at 40°C. there is only a slight production of H₂S. These results show that a temperature of 30° to 34°C. is optimum for H₂S production.

TABLE 3

Representative average results of yearly tests showing consistency of hydrogen sulphide production at 30°C.

TYPE	STRAIN	1927	1928	1929	1929	1930	1930
Aerogenic.....	1	4	4	4	4	4	4
	15	4	4	4	4	4	4
	24	4	4	4	4	4	4
	43	4	4	3	4	4	4
	55	4	4	3	4	3	4
Slightly aerogenic.....	13	tr.	1	tr.	2	1	tr.
	21	3	2	2	3	1	2
	27	1	S	1	1	S	1
	40	2	3	2	2	1	2
	45	3	3	2	3	3	2
Anaerogenic.....	12	—	—	—	—	—	—
	39	—	—	tr.	—	tr.	—
	67	tr.	—	—	—	—	—
	77	*	—	—	—	—	—
	103	*	—	—	—	—	—

* Not included.

Repeated tests at 30°C. upon certain strains show a consistent ability or inability to produce H₂S as evidenced in table 3. Three types are included: aerogenic, slightly aerogenic and anaerogenic. It will be noted that the ability to produce H₂S parallels the ability to produce gas from carbohydrates. Those strains which never produced more than a trace of H₂S never produced gas at any temperature from 25° to 44°C. Furthermore, those strains which were aerogenic at 30°C. but usually anaerogenic at 37° to 40°C. were poor H₂S producers.

DISCUSSION

It is quite evident from the results of this study that the temperature of incubation plays an extremely important rôle in the production of H_2S by *Salmonella pullorum*. Temperatures above $34^\circ C$. not only inhibited production to a marked degree but also gave very irregular results. Therefore, $30^\circ C$. is recommended for routine determinations. It is also of interest to note that this temperature is optimum for the production of gas from glucose as reported by Gwatkin (1928) and Weldin (1929), and confirmed by the writer in unpublished studies. Preliminary studies indicate that this temperature range is also optimum for rate of growth.

Attention is also called to possible variations in temperature at various levels of the ordinary electric laboratory incubator as it is believed that the differences noted by the writer are not unusual.

Only very slight quantitative differences between aerogenic strains were noted at $30^\circ C$. A few strains produced small amounts of H_2S consistently and without exception they were either anaerogenic at $37^\circ C$. or produced only small amounts of gas. A comparison of the amounts of H_2S and the percentage of gas produced from glucose broth showed a direct parallelism for all strains studied. This relationship was found at all temperatures and furthermore the effect of temperature upon H_2S production paralleled that upon gas formation.

Particular attention is called to eleven strains which, upon repeated tests, failed to produce H_2S in any medium regardless of the temperature used. The sources of these strains were: chicks 4, pullet 1, ovaries of reactors 4 and furnished by other laboratories upon request for anaerogenic strains, history unknown, 2. These strains have failed to produce gas from any carbohydrate regardless of incubation temperature. A detailed study of these cultures thus far has failed to show differences from *Salmonella pullorum* except the failure to produce either H_2S or gas and poorer agglutinating capacities toward serum immune to aerogenic strains. Whether or not these strains should be considered as anaerogenic pullorums according to Hadley (1917)

or as a new species remains for future determination; however, the question can be presented for consideration—do they belong to the *Salmonella* or *Shigella* genus? Carbohydrate reactions do not permit their classification as known species of the *Shigella* genus. It is believed that sufficient strains have been used over a long enough period to show that typical strains are consistent in their H_2S producing nature, provided the optimum temperature of $30^\circ C.$ is used.

Studies upon the adaptability of various lead acetate media gave very irregular results. In many cases no H_2S was produced while in other instances growth was inhibited. No doubt the kinds and amounts of peptone, lead salts, etc. were the limiting factors. The results to date show that no medium was equal to Difco lead acetate agar.

SUMMARY

1. An incubation temperature of $30^\circ C.$ is optimum for H_2S production by *Salmonella pullorum*.
2. Temperatures above $37^\circ C.$ inhibit H_2S production.
3. Slight quantitative differences between aerogenic strains were noted at $30^\circ C.$
4. The ability to produce H_2S was a consistent characteristic.
5. Strains which produced little H_2S also produced small amounts of gas from carbohydrates.
6. Strains which produced no H_2S did not produce gas from any carbohydrate.
7. The possibility of a new species is postulated.
8. Difco lead acetate agar is a satisfactory medium with which to show H_2S production.

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A MODIFIED LOEFFLER'S BLOOD SERUM MEDIUM USEFUL IN THE ROUTINE HEALTH DEPARTMENT EXAMINATION FOR DIPHTHERIA AND STREPTO- COCCUS INFECTIONS

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Received for publication August 25, 1930

INTRODUCTION

The shortcomings of Loeffler blood serum for the laboratory diagnosis of diphtheria were brought home to one of us (Medalia) while chief of the laboratory and infectious disease service at a divisional camp and base hospital during the War, when an outbreak of diphtheria occurred. The contacts, sometimes as many as 500 to 750 men (two or three companies), had to be cultured. Since the diagnosis was based upon microscopic examination, the amount of labor entailed is obvious. In addition to the actual labor required in the microscopic examination of so many cultures, there was the difficulty of securing agreement between fairly well trained technicians as to the positive and negative smears. The discrepancy between the findings of the laboratory workers was also met with in the release cultures, mainly because of the scarcity of diphtheria organisms in such cultures.

On the other hand, in the cultivation for typhoid carriers of food handlers, or in cases of epidemic meningitis, where similar routine examination of contacts had to be done, the work, because of special differential media aiding the identification of these two organisms with the naked eye by their characteristic growth on the transparent media, was fairly simple. It, naturally, suggested itself that if a special medium could be found, preferably a transparent medium, which would permit the identification of

C. diphtheriae by the appearance of the colonies on plates, as is true in typhoid and meningitis, or if the selective character of the medium would grow *C. diphtheriae* more luxuriantly or to the exclusion of the other throat organisms, the work in such emergencies would be greatly minimized and possibly better results obtained.

The search for such a special medium was started by one of us (Medalia) in 1920. This was carried on in a research laboratory of the Department of Biology and Public Health of the Massachusetts Institute of Technology. It was at once discovered that in order to duplicate a culture medium, the first prerequisite was the possibility of titrating the culture medium by means of the pH. This resulted in the study of the colorimetric method of titration and the development of the method published by Medalia (1920 and 1922).

The actual development of the special culture medium here reported was accomplished at the Bacteriological Laboratory of the Health Department of the City of Boston, where the facilities for testing such a special medium on a large scale in the routine examination of diphtheria cultures were available. The medium was also tested in a controlled study of cultures obtained at the South Department of the Boston City Hospital and in another controlled study of cultures obtained at the Haynes Memorial Hospital for Contagious Diseases.

This report therefore, deals with three distinct studies: on the one hand, with 2297 cultures obtained by placing the special medium in the same diphtheria outfit which is used by the physicians, with a request to the physicians to plant both media simultaneously. Over this study we had no complete control. We could not positively tell that all such cultures were planted as we requested. On the other hand, the other two studies deal with definitely controlled cultures, which were taken at the South Department of the Boston City Hospital, from newly arrived cases, as well as those cultured for release and others, and at the Haynes Memorial Hospital for Contagious Diseases. The swabs obtained from the nose and throat were placed in a tube containing 2 cc. of nutrient broth. These swabs

were washed in the broth and discarded; fresh sterile swabs were then used for making comparative cultures on the *routine* Loeffler's and the *modified* Loeffler's media. These cultures were incubated under identical conditions, were smeared and stained in exactly the same way, and examined by three and sometimes by four different laboratory workers. The results obtained in these last two studies, we feel, are of definite value.

SPECIAL MEDIA AS FOUND IN THE LITERATURE

The disadvantages of Loeffler's coagulated blood serum medium have been recognized by many laboratory workers ever since Loeffler's classical work on the subject in 1884.

A number of special media, as found in the literature, have been recommended by these workers in an attempt to overcome some of the disadvantages of Loeffler's medium. The more important of these will be briefly discussed here.

Rankin (1911) in an attempt to overcome the necessity for microscopic examination, recommended the use of a medium consisting of three parts of sheep serum, one part bouillon, 1 per cent glucose, 2 per cent potassium sulpho-cyanide 50 per cent solution, and 1 per cent of a 1 per cent solution of neutral red. This is distributed in sterile tubes and coagulated in a slanted position at a temperature high enough to render it sterile.

It is recommended that throat cultures made on this medium be kept in the incubator over night, and if no colonies with red centers are present, that the cultures be returned to the incubator for another twenty-four hours and re-examined. If any colonies with red centers are found they are examined under the microscope to make sure they are *C. diphtheriae*. If no such colonies are present, the cultures are discarded as negative.

The disadvantages of such a method for routine practice are obvious. The element of time alone, would practically eliminate it for purposes of routine work. While some workers believed Rankin's medium to have merit (Coplans, 1911), others (Hanau, 1914) have found it less valuable than Loeffler's medium.

We checked up the medium and found it less selective than Loeffler's. There was no evident inhibition of growth of other

organisms, e.g. the staphylococci, *C. Hoffmanni*, *Streptococcus hemolyticus*, and *Streptococcus viridans*. There was also no characteristic differentiation between the other organisms and *C. diphtheriae* on such plates; i.e., when mixed cultures were planted, it was found impossible to distinguish *C. diphtheriae* from the other organisms; nor was there any apparent inhibition of growth of the organisms that were not *C. diphtheriae*. The chief drawback of this culture medium, however, is the difficulty of its preparation as compared with that of Loeffler's; and, in a comparative study of the two media the former was found not much, if at all, superior to Loeffler's. Such a comparative test was not carried out by Rankin himself. This was done, however, by Hanau (1914), who found the results on Loeffler's medium to be similar to those on Rankin's medium, and in one case he found that *C. diphtheriae* grew on Loeffler's and not at all on Rankin's medium.

Conradi and Troch (1912) made use of a culture medium containing calcium tellurite. No special advantage of this medium over Loeffler's has been found by Hanau (1914) and others sufficient to make it worth while to discard Loeffler's. The same can be said of other special media advanced by their respective proponents, such as the one recommended by Smith (1914) and recently by Douglas (1922).

Practically all of the special media reported in the literature have not been sufficiently checked and tested before they were published. Douglas (1922), for instance, bases his conclusions as to the value of his culture medium on a study of twenty-nine cases (29 cultures) as compared with Loeffler's, where he found nineteen positives on his medium and sixteen on Loeffler's; while Smith (1914) tested his medium on fifty suspected cases of diphtheria against Loeffler's medium and also on Conradi and Troch's medium with the following results: his own medium, ten positives; Loeffler's, eight; while Conradi and Troch's had only six positives. To draw conclusions as to the value of a special culture medium for diphtheria from so few test cultures, is, we feel, not justified.

A number of other special media for *C. diphtheriae* are found in the literature, but as they have not stood the test of time they are not referred to here.

Belding and Fogel (1929) reported, a comparative study by two laboratories of 500 cultures on 77 convalescent diphtheria patients for release. The finding of 43 per cent disagreements as reported by these authors, between their two laboratories is a grave indictment of our means of diagnosis and release of diphtheria cases if that study may be taken as representative of the findings in other laboratories. Belding and Fogel ascribe their main sources of error to the *scarcity* of typical diphtheria organisms and the technical difficulties in taking perfect cultures. According to these authors, the experience of the workers shows a difference in the percentage of disagreements from 10.5 per cent for laboratory directors, to 21 and 30 per cent for experienced and inexperienced technicians respectively.

We feel, therefore, that a culture medium which will grow the *C. diphtheriae* more readily and to the exclusion of the other throat organisms, more especially the staphylococci, as compared with the ordinary Loeffler's medium would eliminate the greatest source of error in diagnosis and release cultures. This, in turn, will help overcome, to a great extent, the endemic prevalence of diphtheria brought about by overlooking cases of diphtheria and by releasing a number of convalescents too soon. Such a culture medium would also tend to avoid the bitter experience met with occasionally, where a child comes home from the contagious hospital supposedly recovered from diphtheria, having been released following three or more negative cultures as required, and a few days later another child in the same household comes down with diphtheria.

The scarcity of growth of *C. diphtheriae* on the ordinary Loeffler's medium and the overgrowth of the cultures by other bacteria is a recognized factor responsible for errors in diagnosis. This scarcity of growth is also referred to by Belding and Fogel as one of the factors responsible for the 43 per cent error found by them. These features are what we hope to overcome with the

culture medium here reported. Such advantages, together with the simplicity of preparation of this culture medium, since it is only a modification of the routine Loeffler's medium, should make it *the* culture medium for use as a routine in laboratory diagnoses and release cultures of diphtheria.

PERSONAL WORK

The routine diphtheria culturing for diagnosis in the City of Boston has been done at this laboratory with Loeffler's blood serum, prepared from pig serum three parts, beef heart infusion one part, to which are added 1 per cent peptone, 1 per cent glucose, and 5 per cent glycerol. The broth is titrated to 0.8 per cent acid.

Since 1927, the pH colorimetric method (Medalia, 1920 and 1922) has been used in this laboratory and the broth standardized to pH 7.2, in place of the titration method.

The serum broth mixture is poured in sterile tubes, slanted and coagulated and sterilized at the same time in the autoclave by heating it at 15 pounds pressure for one hour.

Early in the search for a transparent blood serum culture medium (February, 1919), it was found by one of us (Medalia) that, when Loeffler's blood serum mixture was adjusted to 4.2 per cent alkaline, slanted in tubes, coagulated for three hours at 70° to 80°C. and sterilized in an Arnold sterilizer on three successive days at a temperature between 80° to 85°C., the medium would coagulate and remain clear and transparent, like agar. Attempts at obtaining such a transparent blood serum medium were continued recently by us at the Health Department Laboratory. After many trials, studying different media prepared from beef serum as compared with pig serum with the addition of different amounts of NaOH, the following medium gave the best satisfaction as to transparency and growth:

Pig serum (pH 6.8) 3 parts; beef heart infusion¹ broth (pH 7.2) 1 part; to which are added 4.5 per cent N/1 NaOH, 1 per cent peptone, and 1 per cent glucose. No salt

This mixture gave a reaction of pH 7.6. It was poured into Petri dishes and autoclaved for one hour at 15 pounds pressure, resulting in a solid, clear, transparent medium.

Attempts were made to identify the *C. diphtheriae* colonies by their naked eye appearance from a number of other organisms, such as *C. Hoffmanni*, *Staphylococcus aureus*, the pneumococcus, and *Streptococcus viridans*. It was found, after a long series of cultures, studying the above bacteria on portions of the same plate, that while the streptococci and pneumococci could be identified and distinguished from *C. diphtheriae* because of their characteristic size, the same was not true of the Hoffmann bacillus, or the various staphylococci. Even attempts to identify the colonies of *C. diphtheriae* under the microscope with low power lens were found unsuccessful. At times, we felt that the granular appearance of the colonies would make it possible to do so, yet other organisms at various stages of their growth were found practically similar in appearance, and we were forced to conclude that the diagnosis of the presence or absence of *C. diphtheriae* by the appearance of the colonies in a transparent medium from throat cultures, was not feasible.

Attempts were then made to identify *C. diphtheriae* by making use of a double colored indicator which was found by one of us (Medalia, 1920) to stand sterilization without changing. As early as 1888 Roux and Yersin (1888) found that alkaline broth was made acid by the growth of *C. diphtheriae*, becoming alkaline again after a few days' growth. The acid production by *C. diphtheriae* in an alkaline culture medium of a serum broth mixture containing glucose, with the addition of a double colored indicator, was, therefore, thought by us to be an ideal method of differentiating this organism from other bacteria. Various attempts were made with the transparent culture medium to

¹ Beef heart infusion is prepared from minced beef heart 500 grams water, 1000 cc. kept in ice chest over night, pressed in meat press, made up to 1000 cc. and used for the meat infusion broth.

which were added, before autoclaving, the following indicators of the Clark and Lubs series: brom cresol purple, brom thymol blue, and thymol blue. These were tested in different strengths ranging from 0.05 per cent of a 0.2 per cent alcoholic solution to 0.1 per cent of the same alcoholic solution. The best medium for this study was found to be a serum mixture made with one part beef extract broth, 1 per cent glucose, and three parts pig serum, to which was added 4.5 per cent of N/1 NaOH; reaction pH 7.6; to this was added 0.05 per cent of a 0.2 per cent brom thymol blue alcoholic solution. This was poured in Petri dishes and tubes, coagulated, and sterilized in the autoclave at 15 pounds pressure for one hour. On this medium, too, the *C. diphtheriae* colonies were not any easier to be recognized when grown in mixed cultures with diphtheroids and staphylococci. The attempts to make use of the special culture medium, with or without indicators, for the diagnosis of *C. diphtheriae* by cultural characteristics, without the use of a microscope, was, therefore, abandoned.

In this study the following facts were brought to light:

1. The pig serum, used to prepare the routine Loeffler's medium for the laboratory and which was used in this study, was found to vary from pH 6.4 to 7.0.

2. When the serum was a week old, containing 0.5 per cent chloroform for preservation, and kept in the ice chest, it was found to be at pH 6.4; the addition of 4.5 per cent N/1 NaOH brought the mixture of serum and broth to pH 7.2 (the broth alone was originally pH 7.2). This mixture, when autoclaved and coagulated, instead of being transparent, was opaque.

3. On the other hand, when fresh serum (pH 6.8) was used, the addition of 4.5 per cent N/1 NaOH to the serum broth mixture would bring the mixture up to pH 7.6, which when poured in plates and autoclaved, gave a transparent solid medium.

4. The serum which showed an initial pH of 7.0 instead of pH 6.8 when mixed with broth of pH 7.2, to which mixture was added 4.5 per cent N/1 NaOH, showed a final pH of 7.8 for the mixture. This, when poured into plates and tubes, gave a clear transparent medium, but one which did not coagulate sufficiently for practical use.

It is evident from the facts just described that serum of an unknown pH can not be depended upon to yield the same reaction in the serum broth mixture for Loeffler's medium.

In this study, while using the routine laboratory blood serum medium as a control for the new media studied, the impression gained was that the medium to which 4.5 per cent N/1 NaOH was added and which gave a pH of 7.6 has yielded a much better growth of *C. diphtheriae* than the control, laboratory routine Loeffler's medium. The serum of the latter was not titrated, but was added in the usual proportion, three to one of broth, the broth having been titrated to pH 7.2.

In order to test this "impression," a special study was undertaken for the purpose of obtaining definite data in a large series of cultures, using the "special" medium simultaneously with the routine Loeffler's medium. Before undertaking this study, however, it was thought desirable to test different serum broth media with a view to deciding definitely upon a mixture which would yield the best results.

Six serum-broth media mixtures were prepared:

1. Beef heart infusion broth pH 7.2, 1 part; glucose, 1 per cent; glycerol, 5 per cent; pig serum pH 6.8, 3 parts.
2. Beef *extract* broth, 1 part, in place of the beef infusion broth; glucose, 1 per cent; glycerol, 5 per cent; pig serum pH 6.8, 3 parts.
3. Beef extract broth, 1 part; glucose, 1 per cent; no glycerol; 3 parts of same serum.
4. Beef infusion broth; pH 7.2, 1 part; glucose, 1 per cent; no glycerol; 3 parts of same serum.
5. Beef infusion broth, pH 7.2, 1 part; glucose, 1 per cent; glycerol, 5 per cent; 3 parts of same serum to which was added 4.5 per cent N/1 NaOH, final mixture pH 7.6.
6. Same as no. 5, only substituting beef extract broth for the beef infusion broth.

The composition of the above mixtures was decided upon in order to test whether the heart infusion broth has any advantage over the beef extract broth, the latter being so much easier to prepare; also, to decide whether the glycerol, which has been

used in the routine media, is necessary for its keeping qualities. The addition of the 4.5 per cent N/1 NaOH was made to both the mixture containing infusion broth and the one containing beef extract broth. This was done to decide definitely whether our "impression," that the addition of the 4.5 per cent N/1 NaOH, does make a difference in the growing of the *C. diphtheriae*.

Following a comparative study of cultures made on the six media by planting a mixture of laboratory bacteria (*Staphylococcus aureus*, *Staph. albus*, *Streptococcus*, *Pneumococcus*, with *C. diphtheriae*), it was found that the media containing the NaOH (pH 7.6) with or without the glycerol, were far superior to any of the others.

It was also found that the beef extract broth serum mixture containing the NaOH was in all respects similar to the one made with the beef infusion broth. Consequently, the medium decided upon was that described under no. 6, namely; *beef extract broth, pH 7.2, 1 part, containing 1 per cent glucose, 5 per cent glycerol, pig serum pH 6.8, 3 parts; to which was added 4.5 per cent N/1 NaOH, the final pH of the mixture being 7.6.* We decided upon the use of the pig serum because the beef and horse serum were found unsatisfactory for the preparation of modified Loeffler's because they yielded too soft a medium for routine use. The pig serum we found could easily be obtained fresh from the nearest abattoir by giving one days' notice. It is also more economical as compared with beef or horse serum.

The advantage of this last medium over all the others is that it grows *C. diphtheriae* very luxuriantly and almost in a selective way, while the other media, not containing 4.5 per cent N/1 NaOH, were overgrown by staphylococci, showing only a rare organism of *C. diphtheriae*. On the modified Loeffler's containing the NaOH, *C. diphtheriae* were very abundant and very easy to detect in stained smears.² The glycerol was retained because it prevents the drying of the media when kept for a long time, as occasionally happens in the 73 different Board of Health culture stations and Health Units. This mixture, when put in a

² The stain used in our laboratory is Laybourn's Modification of Albert's Stain.

tube and coagulated, gave an opalescent, semi-transparent medium, which readily withstood the rough use of swab planting, in some respects even better than the routine Loeffler's medium.

That being done, we were ready to undertake the study of testing this medium in the actual culturing of patients in the routine diagnoses or release cultures of the Health Department Laboratory. For that purpose, we placed two culture tubes, of the "special" and "routine" media, together with the swab, in

TABLE 1

Comparison of 2297 diphtheria cultures grown on routine Loeffler's serum and on modified Loeffler's submitted for examination in laboratory outfits by physicians

ROUTINE LOEFFLER'S SERUM, 2297 CULTURES				MODIFIED LOEFFLER'S SERUM, 2297 CULTURES			
Positive	Negative	Suspicious	No growth or contaminated	Positive	Negative	Suspicious	No growth or contaminated
220 9.6%	2008 87.4%	34 1.5%	35 1.5%	306 13.3%	1872 81.5%	32 1.5%	87 3.7%

Of the above number of positive cultures 29, or 1.3 per cent, were found to be practically pure cultures of *C. diphtheriae* on the modified Loeffler's, while only a rare or a few organisms developed on the routine Loeffler's. This was particularly noted in cultures taken for release.

The greater number of "no growth" or "scanty growth" cultures on the modified Loeffler's may be attributed to the fact that frequently physicians failed to plant both sera, using only the accustomed Loeffler's tube. Contaminated cultures were rare, and then occurring in both cultures.

the diphtheria outfits, with a request to the physician to plant both culture tubes at the same time.

This study comprises 2297 routine cultures made by the physicians, or by the medical inspectors and district nurses for diagnosis of suspected cases and of contacts as well as for release cases.

The medical inspectors and nurses on the districts have been taking the release cultures in this study as they usually do and planting them on both culture media. Better control over the work was, of course, possible when the medical inspectors and nurses did the culturing rather than when the private practitioners did it. However, in order to verify these results a comparative

study under absolute control was undertaken, which will be described later.

Table 1 represents a study of the 2297 cultures sent to the Health Department Laboratory by the private practitioners, medical inspectors and nurses. Out of this number there were 220 "positives," or 9.6 per cent, on the routine Loeffler's and 306, or 13.3 per cent, "positives" on the modified Loeffler's, which is higher by approximately 33 per cent. The more important fact, however, which is not brought out by merely studying the figures of the table, is that the modified Loeffler's, because of the more luxuriant growth of *C. diphtheriae* and the ease of detecting the organism microscopically, enabled the diagnosis to be made in a very short time, while it took a considerably longer time and was much more difficult to reach a conclusion when examination was made of the routine Loeffler's. In fact, out of the total number of positive cultures, there were on the modified Loeffler's 29, or 1.3 per cent, which were practically pure cultures of *C. diphtheriae*, while only an occasional or rare organism was found on the routine Loeffler's. This was particularly true in the cultures taken for release.

The number of "suspicious" cultures was about the same on both media. The greater number of "no growth" or "scanty growth" on the modified Loeffler's may be accounted for by the fact that the physicians failed to plant both media, using only the routine media to which they were accustomed, and which had the label, while the modified Loeffler's did not have such a label.

There rarely was a case where the *C. diphtheriae* was positive on the routine Loeffler's and negative on the modified medium.

Another important finding in this study of the 2297 cultures was the ease with which the streptococcus grows on this medium. Since the finding of streptococci has to be reported by the laboratory to the physicians, a careful record was kept of such findings parallel with the finding of *C. diphtheriae*. The results were astonishing, namely: only 11, or 0.5 per cent, of the total number showed the presence of streptococci on the routine Loeffler's, while 133, or 6.2 per cent, showed the presence of

streptococci as a predominating organism on the modified Loeffler's.

As already referred to, the results of the study just described, dealing with comparative cultures obtained at the Laboratory in a routine way, were not considered by us to be entirely satisfactory because of our inability to control the culturing. There might, for instance, have been cases where some practitioners, in

TABLE 2

Comparison of 2297 cultures of table 1 grown on the routine Loeffler's and the modified Loeffler's media with relation to their respective ability to grow the streptococcus

ROUTINE LOEFFLER'S SERUM, 2297 CULTURES—STREPTOCOCCUS	MODIFIED LOEFFLER'S SERUM, 2297 CULTURES—STREPTOCOCCUS
11 0.5%	133 6.2%

It will be noted that modified Loeffler's permits the growth of a much larger percentage of streptococcus cultures than the routine Loeffler's.

TABLE 3

Comparison of 494 diphtheria cultures planted simultaneously on two media in the laboratory

ROUTINE LOEFFLER'S MEDIA, 494 CULTURES				MODIFIED LOEFFLER'S MEDIA, 494 CULTURES			
Positive	Negative	Suspicious	No growth or contaminated	Positive	Negative	Suspicious	No growth or contaminated
20 4.04%	457 92.5%	9 1.8%	8 1.7%	85 17.2%	372 75.4%	19 3.8%	18 3.6%

This study was controlled at every step.

It will be noted that the modified Loeffler's media grew over four times as many "positives" as the routine Loeffler's. The growth was far more abundant and in some instances almost a pure culture of *C. diphtheriae*.

spite of the request to plant the two culture media alike, planted one with the swab obtained from the throat and the other with one obtained from the nose. Although we have tried always to check up in those cases, when a positive was found on the modified Loeffler's and a negative on the routine Loeffler's, still we were not satisfied that this could be considered an absolutely controlled piece of work. We, therefore, undertook the following study.

The cultures were made at the South Department of the Boston City Hospital. The swabs, one from the throat and one from the nose, were placed in a tube containing 2 cc. of nutrient broth. These swabs, when brought to the Laboratory in the broth, were carefully washed in this broth and removed from it and discarded. A fresh sterile swab was used to plant each of the routine and modified media, properly labeled and incubated over night. They were then smeared, stained, and examined

TABLE 4

Comparison of 189 diphtheria cultures planted simultaneously on three media in the laboratory

LABORATORY LOEFFLER'S MEDIA, 189 CULTURES				HOSPITAL LOEFFLER'S MEDIA, 198 CULTURES				MODIFIED LOEFFLER'S MEDIA, 189 CULTURES			
Positive	Negative	Suspicious	No growth or contaminated	Positive	Negative	Suspicious	No growth or contaminated	Positive	Negative	Suspicious	No growth or contaminated
7	179	2	1	20	165	3	1	34	149	4	2
3.7%	94.7%	1.1%	0.5%	11.0%	87.0%	1.5%	0.5%	18.1%	78.7%	2.1%	1.1%

This is another controlled study on three different media.

The modified Loeffler's again showed over four times as many "positives" as the routine Loeffler's.

The Hospital media to which is added NaOH until it reaches a definite pink to phenolphthalein made a better showing but is not up to the modified Loeffler's in character of growth nor in number of "positives".

microscopically by at least three different Laboratory workers, and sometimes four. Table 3 gives the result of this study.

It will be seen that this study was controlled at every step, namely, the broth was used merely to wash the nose and throat swabs; fresh sterile swabs were used to plant from the broth onto the culture media, which were studied. The media were incubated under exactly the same conditions, stained and examined in a similar way.

A second controlled, comparative study was also made by using, not only the routine Loeffler's medium of the Board of Health Laboratory alongside the new medium, but also by using

the hospital Loeffler's serum. Table 4 gives the result of this study, which was carried out in exactly the same manner described for the one reported in table 3.

A critical examination of table 3, which represents the controlled study on the two media, shows that there were 494 cultures, of which 20, or 4 per cent, were found positive on the routine medium, as against 85, or 17.2 per cent, positives on the modified Loeffler's. The "suspicious" cultures in this study were 9, or 1.8 per cent, on the routine, and 19, or 3.8 per cent, on the modified Loeffler's media.

The streptococcus findings were not included in this study. It was limited to diphtheria only. The positives on the modified Loeffler's, according to our findings, are over four times those found on the routine Loeffler's, the significance of which speaks for itself.

Table 4, which represents another controlled study on three different media instead of two, shows again the value of the modified Loeffler's, medium for the growth of the *C. diphtheriae*. Here we find 7 positives, or 3.7 per cent, in a total of 189 cultures, on the routine media, while the modified Loeffler's shows 34, or 18 per cent, positives, which is again $4\frac{1}{2}$ times as many positives as on the routine. The "suspicious" findings here too were twice as many as on the routine media. The hospital media made a better showing in this study than the routine: 20 positives, or 11 per cent, out of the total 189, as against 3.7 per cent on the routine, and 18.1 per cent on the modified media. Upon investigation we found that the hospital media contained NaOH, which was added by testing the regular pig serum-broth mixture to a drop of phenolphthalein on a porcelain plate. Enough normal NaOH was added so that the indicator turned a definite pink. In case too much NaOH had been added, HCl was used until the desired pink was obtained.

The method of titration just described, as followed in the preparation of the hospital medium, is unreliable because this method does not yield the same pH for the media prepared at different times. However, even at that, the results of growing *C. diphtheriae* were better than without the addition of the

NaOH. The use of the pH method in the preparation of any media is now recognized as the standard reliable way of obtaining a culture medium which can be duplicated at all times.

Another study was carried out with cultures obtained at the Haynes Memorial Hospital for Contagious Diseases by a student of Boston University, Elizabeth Goodman, to whom we are indebted for this work.

The swabs were placed in 2 cc. nutrient broth. Thesecretions were washed off them and the original swabs discarded. Fresh sterile swabs were used to make cultures on the routine Loeffler's and the modified Loeffler's. They were incubated, stained, and examined in identically the same way. The work as a whole was

TABLE 5
Comparison of 176 diphtheria cultures planted simultaneously on two media in the laboratory

ROUTINE LOEFFLER'S MEDIA, 176 CULTURES			MODIFIED LOEFFLER'S MEDIA, 176 CULTURES		
Positive	Negative	Occasional or suspicious	Positive	Negative	Occasional or suspicious
4 2.2%	167 95%	5 2.8%	15 8.5%	156 88.7%	5 2.8%

This is another controlled study carried out in a different hospital by another worker.

It will be noted that here too the modified Loeffler's showed four times as many "positives" as the routine Loeffler's.

done in a similar manner to that employed in the studies referred to in tables 3 and 4.

Table 5 gives the results of this study. There were 176 cultures planted simultaneously on the two media. There were four positives, or 2.2 per cent, on the routine Loeffler's medium, while there were 15 positives, or 8.5 per cent, on the modified Loeffler's. In this study, as in the other two controlled studies, the modified Loeffler's showed more than four times as many positives as the routine Loeffler's. Here, too, the striking factor, however, was not merely the greater number of positives in the modified Loeffler's, but the greater abundance of growth as compared with that on the routine Loeffler's, which means

greater ease of diagnosis, in the case of the use of modified Loeffler's.

In the light of our findings, showing the great sensitiveness to changes in pH of culture media of the bacteria here considered, *C. diphtheriae* and particularly the streptococci, we feel that the methods used in some laboratories, of determining the pH by merely adding a few drops of phenol red to a solution and guessing the pH by the change of color produced in the solution is unreliable. We found brom thymol blue to be the best and most reliable indicator and the use of the method with the color standards as described by Medalia (1920 and 1922) almost as easy as the indiscriminate use of a few drops of phenol red.

The use of brom thymol blue with the color standards and the "comparator" block permits the definite determination of the pH within 0.1 of a pH; but what is more important, the method permits the use of additional tubes to offset the color of the culture medium and also to determine whether the particular indicator can be used at all.

We found, for instance, that phenol red does not permit the matching of color in the preparation of the Loeffler's or the modified Loeffler's media, possibly because of the "protein" or "salt" error in the serum. This difficulty is overcome by the use of brom thymol blue. The importance of definitely determining the pH in culture media is brought home by this study since, in such a highly buffered medium as we have used, the difference of 0.4 pH between the two media has made such a marked change in the growing of *C. diphtheriae*, yielding four times as many positives and growing it almost in a selective way, while the streptococci grew more than twelve times as often as on the routine media.

SUMMARY

A blood serum medium has been described, which is almost a selective culture medium for *C. diphtheriae*. It is easy to prepare, since it is only Loeffler's medium, modified by the addition of 4.5 per cent N/1 NaOH yielding a final pH of 7.6 using brom thymol blue as an indicator.

The possibility of checking the reaction in the final mixture by the pH "colorimetric" method (Medalia, 1920 and 1922) makes this culture medium easy to duplicate and yields uniform results.

In definitely controlled studies (tables 3, 4, and 5), the positive findings on the modified Loeffler's medium are more than four times as many as on the routine medium. The ease with which this modified Loeffler's grows *C. diphtheriae*, almost in a selective way, permits the examination to be made in a much shorter time and the positives are more readily discernible.

From the standpoint of the Health Department Laboratory examination, this culture medium has also been of great value because of the ease with which it grows the streptococcus; in a study of 2297 cultures (table 2), where on the routine Loeffler's medium only 0.5 per cent streptococci were found predominating, on the modified Loeffler's, 6.2 per cent were found.

The modified Loeffler's culture medium here described, is therefore, we feel a very valuable one for the routine examination of diphtheria and streptococcus organisms, and we especially recommend it to Health Department Laboratories for this purpose.

In concluding, we wish to express our appreciation and thanks to Dr. Francis X. Mahoney, Health Commissioner, City of Boston, to Professor Hans Zinsser of Harvard, Dr. D. L. Belding of Boston University, Dr. Edwin H. Place of the Contagious Department of the Boston City Hospital, Dr. Conrad Wesselhoef of the Haynes Memorial Hospital for Contagious Diseases, and J. Etta Mullen, Bacteriologist, Health Department, through whose kindly advice and sympathetic cooperation this study was made possible.

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PLATE 1

Swabs were taken from nose and throat washed in 2 cc. of bouillon and cultured simultaneously with fresh sterile swabs on routine Loeffler's media and modified Loeffler's media.

FIG. 1. Microphotograph of the growth on the routine Loeffler's media showing occasional *C. diphtheriae* with a large number of other organisms both staphylococci and bacilli.

FIG. 2. Microphotograph of the same culture as figure 1 grown on the modified Loeffler's media. This proved to be almost a pure growth of *C. diphtheriae*.

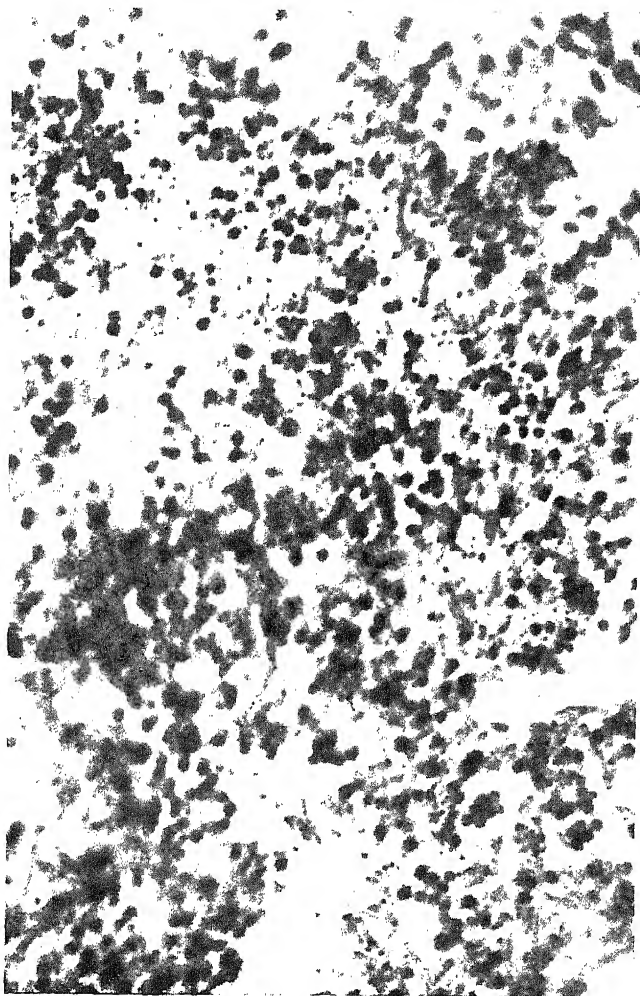


FIG. 1

(L. S. Medalia, et al.: Modified Loeffler's blood serum medium.)

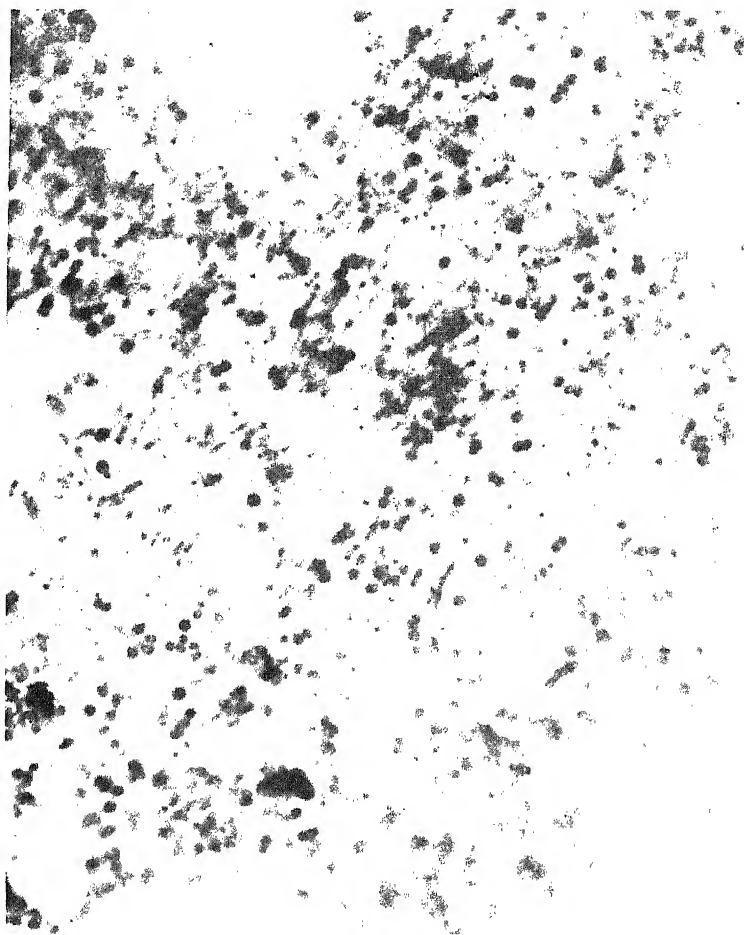


FIG. 2

(I. S. Medalia, et al.: Modified Loeffler's blood serum medium.)



CHARLES KRUMWIEDE (1879-1930)
President, Society of American Bacteriologists, 1920

Charles Krumwiede

1879-1930

Doctor Charles Krumwiede was born in New York City, September 9, 1879. He graduated from Columbia University in 1902 and from the College of Physicians and Surgeons in 1905. He then entered the French Hospital as an interne and later became assistant pathologist there.

In 1909, a fund of \$10,000 was voted by the City of New York for a study of the importance of the bovine type of bacilli in human infection. Dr. Krumwiede, along with several others, was appointed under this appropriation as an assistant bacteriologist in the laboratory of the Department of Health.

The investigation was carried on under the general supervision of Dr. Park. In his portion of the work, Dr. Krumwiede showed remarkable ability and no small part of the important results obtained from the investigation was due to his efforts. His services were estimated so highly that he was later appointed one of the assistant directors of the Bureau of Laboratories of the Department of Health.

In 1910 he was appointed an instructor in bacteriology in the New York University and Bellevue Hospital Medical College. He later became Associate Professor of Bacteriology and Immunology and in 1930 he became a full professor. He served as President of the Society of American Bacteriologists in 1920.

He impressed those who knew him with his keen intellect, technical knowledge and absolute honesty. He was an excellent teacher. He held the attention of the students and led them to think for themselves insofar as that was possible.

He assisted with Drs. Park and Williams in the editing of "Pathogenic Microorganisms" and in 1929 became joint editor.

He wrote many articles on bacteriological and immunological subjects, some of them of great importance. Among the subjects

to which he contributed were the improvement of diagnostic media for the detection of cholera vibrios and the use of green dyes which had a selective inhibitive action on Gram positive bacteria. The use of media containing brilliant green has been especially useful in distinguishing colonies of typhoid bacilli from those of other fecal bacteria.

He was deeply interested in the study of the paratyphoid enteritidis group. Another subject which attracted his attention was the improvement of the method of typing pneumococcus in sputum. Because of this he developed his precipitin test, which is very generally in use. He was greatly interested in the study of precipitins and agglutinins, and he was one of the first to develop and use the quick microscopic slide agglutination.

Together with his associates in the laboratory, he published a monograph on the general subject of agglutinin absorption. His last important work was on the subject of psittacosis. Together with his associates he proved that it was due to a filtrable virus. His observations were finished and published before the work of other laboratories coming to the same conclusion was printed.

Those who came in contact with him professionally were impressed by his depth of knowledge and by his excellent judgment. Those of us who were his associates in the laboratory had learned to depend upon him and we miss him both as an associate and as a friend. He had the respect and affection of all who came in contact with him.

WILLIAM H. PARK.

A QUANTITATIVE STUDY OF THE RESPIRATION OF STAPHYLOCOCCUS CULTURES LYSED BY BACTERIOPHAGE

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Received for publication, July 10, 1930

INTRODUCTION

Most of the experimental work intended to demonstrate that the bacteriophage and the filterable viruses have an independent metabolism has been done on material in which the agent is probably in a metabolically inactive state. Attempts to demonstrate respiration by various methods have yielded conflicting results. Kempner (1927), working in Warburg's laboratory and using the method described for the present work, reported that sera taken from chickens with fowl-plague had a demonstrable respiration whereas normal control sera did not respire. Using the same method on hog cholera and measles sera, Irvine-Jones and Schoenthal (1929) were unable to detect respiration. Bronfenbrenner (1924-1926) was unable to detect CO_2 production by filtered bacteriophage. Bronfenbrenner and Reichert (1926) measured the oxygen consumption and CO_2 production of tissues containing herpes virus and rabies virus aged in glycerol and found that the rate of respiration did not differ materially from that of normal tissues similarly treated. Bronfenbrenner (1928) states that the rate of respiration of a bacterial culture being acted upon by bacteriophage is the same as that of a control culture when allowance is made for the "higher rate of multiplication of bacteria in the presence of the bacteriophage." We have been unable to find in the literature any record of the experimental basis for this statement and certain of the results of the present work directly contradict it.

It is not improbable that the bacteriophage may be metabolically dependent upon the susceptible bacteria, and, therefore, may become inactive and cease to respire when some activating part of the bacterial cell has been used up. The experiments described below were undertaken with the purpose of studying the bacteriophage in its most active state. They represent quantitative measurements of the respiration when a large quantity of bacteriophage is added to a culture of rapidly growing staphylococcus and produces prompt and almost complete lysis of the organisms.

METHOD

The method of measuring respiration is essentially the same as that first described by Warburg (1926). Microrespirometer flasks have been constructed with a side arm to hold the broth containing bacteriophage and a central cup in which is placed 5 per cent solution of KOH to absorb the CO₂ formed. Plain broth is inoculated with two drops of a broth culture of *Staphylococcus aureus* (strain JB2) and 3.0 cc. are pipetted into the respirometer flask which is then connected with the manometer and shaken gently in a water bath at a temperature of 37.5°C. Accurate determinations of the rate of respiration of an actively growing culture may be made in periods of ten minutes. The stopcocks are opened, admitting air, after each reading.

In order to obtain uniform results fresh bacteriophage must be prepared before each experiment. The bacteriophage is passed through several generations of rapidly growing bacteria so that its virulence is at a maximum, and then filtered. Before each experiment a loopful of this stock bacteriophage is added to a six-hour culture of staphylococcus and incubated over night. The culture so treated is then diluted 1:10 with broth and 0.3 cc. is placed in the side arm of the respirometer. When the bacteria are multiplying at the maximum rate as determined by control experiments the bacteriophage is added. By this method prompt action is obtained.

Bacterial counts are done in order to compare the number of bacterial cells with the rate of respiration of the whole culture,

and to follow the process of destruction of the bacteria closely and accurately. A special counting chamber 0.02 mm. deep is used. A drop of the culture is removed from the respirometer flask, before and after each determination of the rate of respiration, and allowed to run into the counting chamber. When over 500 million bacteria per cubic centimeter are present the culture should be diluted 1:10 by means of a diluting pipette. When the number of intact cells falls below one million per cubic centimeter the method of plating is used.

RESPIRATION OF NORMAL CULTURES

The curves of figure 1 depict the respiratory activity of the staphylococcus growing in the microrespirometer without the addition of bacteriophage. The upper curve represents the rate of oxygen consumption of the culture in cubic millimeters per cubic centimeter of culture per hour. The lower curve shows the rate of absorption of gas measured without alkali in the central cup to absorb the CO_2 ; that is, the difference c.mm. O_2 - c.mm. CO_2 . The true respiratory quotient cannot be calculated from these curves because part of the CO_2 formed dissolves in the broth and combines with ammonia and other alkaline products of metabolism. The secondary wave in the lower curve has not been adequately explained. It may be due to the accumulation and subsequent liberation of CO_2 from the broth.

The dotted line represents the growth curve, and a comparison of this curve with the curve of oxygen consumption will show that the respiration of a young actively growing culture is an accurate measure of the number of bacteria present. As the culture ages, some of the bacteria die and the rate of respiration gradually decreases.

RESPIRATION DURING THE ACTION OF THE BACTERIOPHAGE

After several experiments it became evident that the action of the bacteriophage on organisms in the microrespirometer was somewhat different from its action in a culture tube. Since the broth is kept in intimate contact with air in the respirometer flask by means of gentle shaking, the oxygen tension of the entire

culture is undoubtedly higher than that of a test tube culture where only the upper layer of broth is in equilibrium with atmospheric oxygen (cf. Avery and Morgan (1926)). Arloing, Langeron and Sempe (1925) found that shaking in the open air had no destructive effect upon the bacteriophage. In fact, from our experience, these conditions seem to favor its action. In the respirometer, lysis is complete in three to five hours, while in an ordinary culture tube the process requires eight to twelve hours. An-

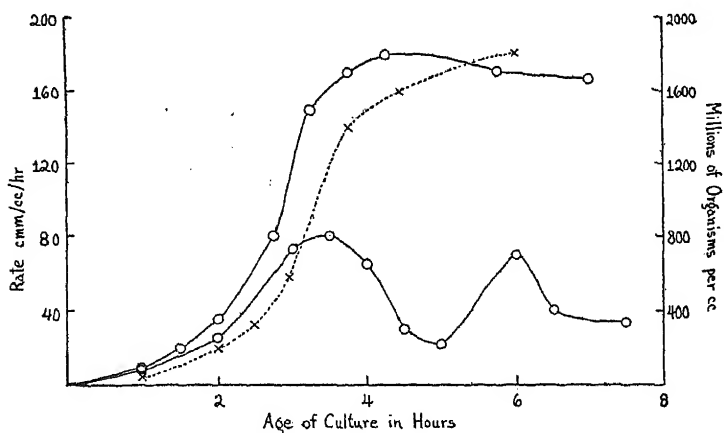


FIG. 1. RESPIRATION RATE AND GROWTH OF A NORMAL CULTURE OF STAPHYLOCOCCUS

The upper solid line represents the rate of oxygen consumption in cubic millimeters of O₂ per cubic centimeter of the broth culture per hour. The lower solid line represents the difference, c.mm. O₂/cc./hour minus c.mm. CO₂/cc./hour. The height of the upper curve minus the height of the lower curve gives the rate of CO₂ formation by the culture. The broken curve represents the number of bacteria per cubic centimeter as determined by direct counts.

other noteworthy difference is that there is a greater tendency to agglutination of the organisms in the microrespirometer. This may be due to the mechanical effect of shaking.

When a large amount of bacteriophage is added to a culture in the microrespirometer, the first effect to be noted is that the bacteria stop multiplying almost immediately. In a few minutes the cocci group together in small clumps of five or ten organisms. Lysis begins and proceeds rather rapidly along with the process of agglutination, so that gelatinous masses containing several intact

cells may be seen under the microscope. Accurate counts are difficult during this stage. Finally the process proceeds to com-

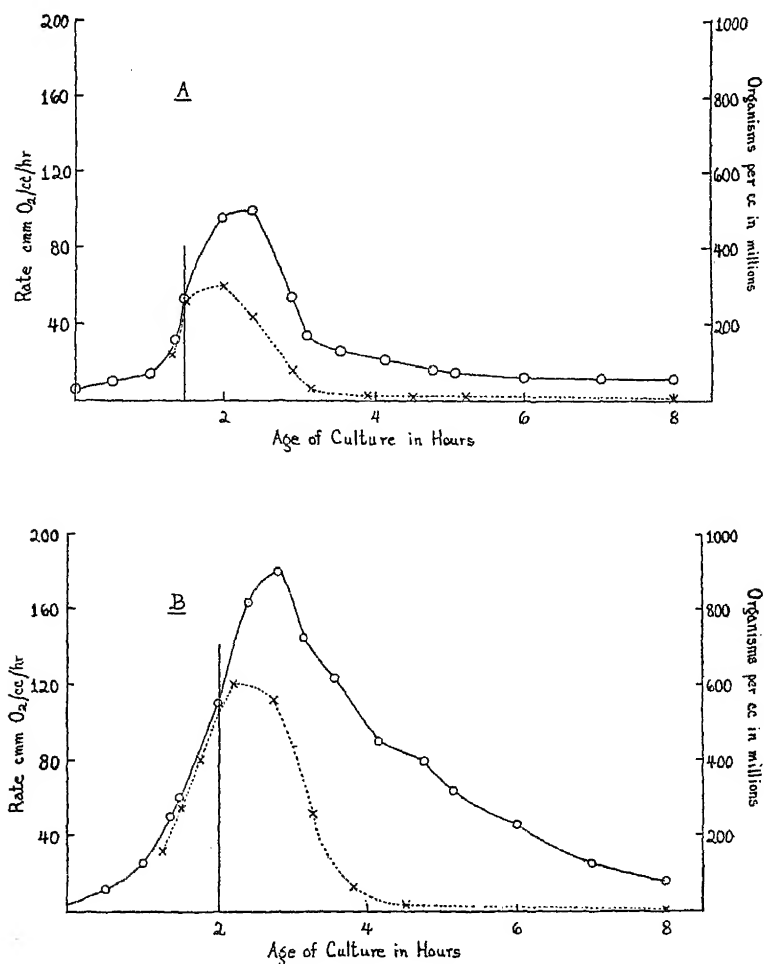


FIG. 2, A, B. EFFECT ON THE RESPIRATION PRODUCED BY ADDING BACTERIOPHAGE

The vertical lines represent the points at which the bacteriophage was added, at the beginning of maximum growth in A and near the end of maximum growth in B. The solid lines show the rate of oxygen consumption in cubic millimeters per cubic centimeter of culture per hour. The broken lines show the number of intact bacterial cells.

plete clearing of the culture and no intact cells may be seen in the counting chamber.

The curves of figure 2 represent the results of two series of experiments in which the rate of oxygen consumption is compared with the number of intact cells in suspension. Each point on these curves represents the average of three experiments. The solid lines show the rate of oxygen consumption of the cultures and the dotted lines the number of intact bacteria as determined

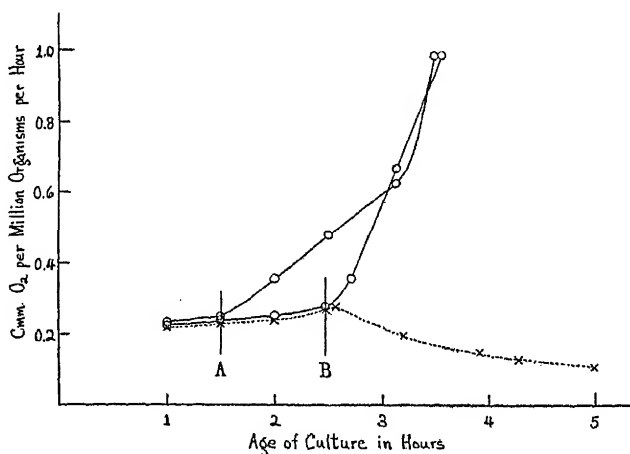


FIG. 3. RATE OF RESPIRATION IN TERMS OF THE NUMBER OF ORGANISMS

Solid lines represent the respiration after adding bacteriophage in two series of experiments A and B. Broken line the rate of respiration of a normal culture without bacteriophage. Values calculated as cubic millimeters of oxygen consumed by one million organisms in an hour.

by direct counts. In the series represented by curve A the bacteriophage was added just after the beginning of the phase of maximum rate of growth, and in the series B near the end of this phase.

The curves of respiration after the addition of bacteriophage may be divided into three stages:

1. The stage of invasion which lasts twenty to forty minutes. In this stage, counts made ten minutes after the addition of the bacteriophage show that the number of bacteria is practically

- the same as before the bacteriophage was added. On the other hand, the rate of respiration has continued to increase.
2. The stage of activity during which the bacterial count and the respiration both fall rapidly, the former more rapidly than the latter.
 3. The stage of residual respiration during which the respiration is sustained for at least ten hours. In this stage the number of intact cells can only account for a small fraction of the observed respiration.

The curves in figure 3 are included to show more clearly the relation of oxygen consumption to the number of intact bacteria in suspension in the culture. They are calculated from the data for figure 2 and represent the rate of oxygen consumption per million organisms per hour. The dotted line represents the rate per million organisms calculated from the data on control cultures of staphylococcus without bacteriophage. From a glance at these curves it may be seen that the rate of respiration appears to rise almost immediately after the addition of the bacteriophage. These observations on the effect of the bacteriophage on the respiration of the staphylococcus are practically identical with the results of experiments done in this laboratory a year ago by Harris (unpublished). However, at that time no attempt was made to determine the numbers of intact or viable organisms. Harris also showed that when bacteriophage alone or a freshly inoculated culture containing bacteriophage is used, no respiration is measureable.

That the residual respiration is not due to the multiplication of resistant forms may be shown by plating experiments. If a loopful of the culture is plated out during various parts of the stage of activity, massive growth is obtained at first and this decreases to a few colonies only as the lysis nears completion. During the stage of residual respiration only one or two colonies at the most may be obtained from a loopful of the lysed culture and the plating of five drops may give only one or two colonies. In other words the culture has become practically sterile as far as intact, viable or resistant organisms are concerned; yet it respirees at approxi-

mately the same rate as a normal culture containing 50 to 100 million organisms per cubic centimeter. Resistant forms do not appear in the lysed cultures until after forty-eight to seventy-two hours.

Often, after the culture in the microrespirometer has cleared, a single macroscopic white flake of bacterial substance remains. That this does not consist of intact or viable organisms may be shown by microscopic examination and plating. The flake is apparently a gelatinous mass of lysed bacterial cells. No organisms will grow when one of the flakes is spread upon blood agar. In order to determine whether or not the residual respiration is due to these gelatinous masses of bacterial substance, several of them were removed, washed rapidly in broth, and placed in sterile broth in separate respirometer flasks. It was found that this broken down bacterial substance did respire, but at a rate only 20 per cent of the total residual respiration of the lysed culture. It is obvious that during the brief process of washing all the adherent bacteriophage was not removed.

If the lysed bacterial culture is removed by means of a capillary pipette to another respirometer flask, care being taken not to include any of the mass of gelatinous substance, the rate of respiration of this fluid, which is practically free of microscopically visible particles, is about 75 per cent of the total residual respiration. This respiration, plus the respiration of the coagulum, accounts for most of the observed residual respiration. This experiment shows that the respiration is not due to resistant bacteria adhering to the glass of the respirometer flask.

Experiments to demonstrate respiration of filtered cultures containing bacteriophage were unsuccessful. Cultures in the stage of residual respiration were filtered and placed in respirometer flasks, a total time of four hours having elapsed since the addition of the bacteriophage to the culture. The observed rate of respiration was only about 3.0 c.mm. an hour, a figure very close to the limits of experimental error; and even this respiration continued for only an hour. Filtering seems to destroy the respiratory activity of lysed bacterial cultures.

PROOF OF THE PRODUCTION OF CO_2 BY LYSED CULTURES

The possibility that the observed oxygen consumption in the stage of residual respiration may be due to oxidation of the products of bacterial disintegration must be considered. If this were the case, we should not expect to be able to detect the production of CO_2 , since the oxidation of complex carbon compounds to CO_2 occurs at ordinary temperatures only in the presence of special enzymes or inorganic catalysts. We believe, therefore, that any process occurring at low temperatures in which 0.5 to 1.00 volume

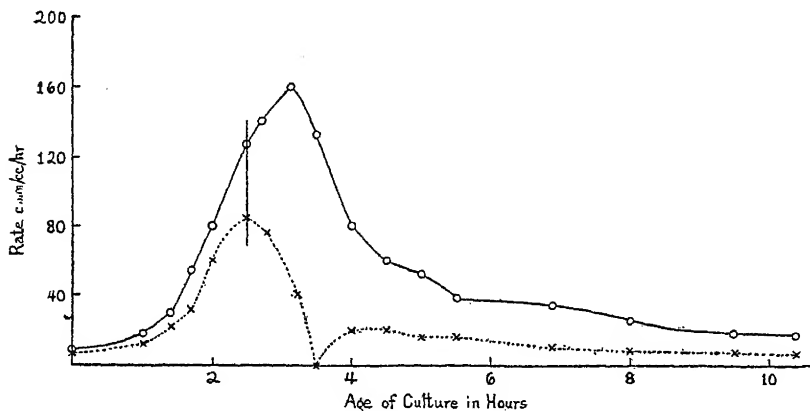


FIG. 4. The upper curve represents the rate of oxygen consumption per cubic centimeter of culture per hour. The lower curve is the difference between O_2 consumed and CO_2 evolved. Vertical line shows point at which bacteriophage was added.

of CO_2 is given off for each volume of oxygen consumed must be considered true respiration until proved to be otherwise.

Figure 4 gives the results of two series of experiments similar to those previously described. The solid curves represent the rate of oxygen consumption of the culture in cubic millimeters per cubic centimeter of culture per hour. The dotted curves represent the rate of gas exchange measured without alkali to absorb the CO_2 . If no CO_2 were evolved the dotted and solid curves should correspond.

It is worthy of note that the curve of $\text{O}_2 - \text{CO}_2$ begins to descend a short time after the addition of bacteriophage, whereas the curve

of O_2 continues to rise. This indicates that the addition of bacteriophage to the culture causes an increase in the evolution of CO_2 . When the bacteriophage is added near the end of the period of maximum growth, the curve for $O_2 - CO_2$ shows a secondary wave similar to that observed in the cultures of normal staphylococcus. Earlier addition of bacteriophage entirely prevents the appearance of this wave. Differences in pH are negligible and therefore do not account for this observation.

EFFECT OF THE CONCENTRATION AND RATE OF GROWTH OF THE
BACTERIA AT THE TIME OF ADDING BACTERIOPHAGE

Since the rate of oxygen consumption of a young culture of staphylococcus is an accurate measure of the number of living

TABLE 1

AGE OF CULTURE	RATE ON ADDING BACTERIOPHAGE	RATE OF RESPIRATION AT TIMES SHOWN AFTER ADDING BACTERIOPHAGE			
		40 minutes	2 hours	4 hours	8 hours
hours		<i>c.mm. per cc. per hr.</i>	<i>c.mm. per cc. per hr.</i>	<i>c.mm. per cc. per hr.</i>	<i>c.mm. per cc. per hr.</i>
2	14	35	8	4	3.5
2 $\frac{1}{4}$	36	83	16	10	6
3	78	125	34	28	17
3 $\frac{1}{2}$	120	160	35	30	17
4	140	168	118	40	19
5 $\frac{1}{2}$	180	166	74	30	19

bacteria present, the respiration curve furnishes a convenient means of determining the amount and rate of growth. The figures in table 1 give the results of an experiment in which the same concentration of bacteriophage was added to cultures in various stages of growth. The numbers in the first column give the age of the culture in hours at the time of adding the bacteriophage. The second column gives the rate of oxygen consumption just before the bacteriophage was added. The last four columns show the rate of oxygen consumption of the cultures at various times after adding bacteriophage.

In every case except that represented by the last line of the table, the rate of respiration increases after adding bacteriophage

to the culture and the amount of increase in rate is less in the older cultures. However, as previously pointed out the number of intact bacteria does not appreciably increase during this period. When the bacteriophage is added in the early stages of growth, the residual respiration seems approximately related to the initial number of respiring bacteria, as shown by the first three lines of the table. When the bacteriophage is added during the period of maximum rate of growth or after this period, the residual respiration reaches practically the same level in all cases, and is not related to the initial concentration of bacteria.

If the residual respiration were due to the bacteriophage, we might expect that it would be greatest when the agent acted upon

TABLE 2

DILUTION OF BACTERIOPHAGE	RATE OF RESPIRATION AT TIMES SHOWN AFTER ADDING BACTERIOPHAGE					
	15 minutes	30 minutes	1 hour	2 hours	4 hours	8 hours
	<i>c.mm. per cc. per hr.</i>	<i>c.mm. per cc. per hr.</i>	<i>c.mm. per cc. per hr.</i>	<i>c.mm. per cc. per hr.</i>	<i>c.mm. per cc. per hr.</i>	<i>c.mm. per cc. per hr.</i>
Undiluted	160	141	82	30	23	18
1:5	146	150	107	60	25	18
1:25	153	170	134	82	33	19
1:125	126	136	139	96	48	21
1:600	130	145	153	160	57	23
1:3,000	145	150	156	150	78	23

a culture growing at the maximum rate; that is when the agent is supposedly most active. The experimental data shown here are inconclusive on this point and more extensive measurements will be necessary.

EFFECT OF THE CONCENTRATION OF BACTERIOPHAGE

The figures in table 2 show the effect of adding various dilutions of the same tube of bacteriophage to cultures in approximately the same stage of growth, respiring at the rate of 130 to 140 c.mm. O₂ per cubic centimeter per hour. The smaller the quantity of bacteriophage added, the more sustained is the respiration but the initial rise is sharper when large amounts are added. The fall of respiration during the stage of activity seems to be quite defi-

nately related to the rate of destruction of the bacteria. The residual respiration in all cases eventually reaches the same level.

DISCUSSION

Unfortunately it cannot be said that the experiments described in this paper conclusively prove that the bacteriophage respire. The results seem to indicate that respiration of the bacteriophage, if it occurs at all, only takes place during the time when the bacteria are being destroyed and for several hours afterward. In other words, the bacteriophage only respire when some constituent of the bacterial cell completes in it the equipment necessary for respiration. The results do, however, make it seem certain that the observed rate of respiration of the lysed culture, even though it is considerably less than that of a normal culture, cannot be accounted for solely by the number of intact viable organisms present.

The possibility that the broken up bacteria, or a combination of these with the bacteriophage, respire must be considered. Several instances are known in which respiration occurs after the integrity of the cell has been destroyed. Red blood corpuscles continue to respire after hemolysis, and sea urchin eggs respire after they have been broken up by grinding. Thermostable tissue residues which exhibit true respiration have also been described. The oxidation of organic compounds to CO_2 and water in the presence of atmospheric oxygen and an inorganic catalyst such as ferrous salts or charcoal is a well known phenomenon, but it is improbable that we are dealing here with any such inorganic system as this.

In none of the experiments with various concentrations of bacteriophage acting on bacteria in various stages of growth was any evidence found that bacteriophage stimulates the rate of division. When large amounts of bacteriophage act on a rapidly growing culture, growth stops almost immediately. Otherwise the bacteria multiply at a diminished rate. Although the rate of respiration continues to increase after the addition of bacteriophage the slope of the curve is usually slightly less than that of a normal culture, and never greater. If, as reported by Bronfenbrenner (1928) the

bacteriophage stimulates growth and thus produces a proportional increase in the rate of respiration of the culture, we would expect the slope of both the respiration curve and the growth curve of a culture with bacteriophage to become greater than the slope of the curve for a control culture. This is the opposite of the observations reported here.

The results of the present work seem to exclude the theory that the lysis of bacteria by the bacteriophage is a process of simple autolysis. In order that cells may be digested by their own enzymes they must first be killed. If the action of the bacteriophage proceeded in this fashion we should expect that the respiration would stop first and the lysis of the bacteria occur later. A few experiments on the respiration of the pneumococcus, done several months ago, showed that a fall in the rate of respiration per million cells preceded the beginning of autolysis of these organisms. In the action of bacteriophage on susceptible bacteria the lysis of the cells precedes the fall in respiration of the culture and the respiration continues after lysis is complete.

SUMMARY

1. Evidence is presented which indicates that, when a large amount of bacteriophage actively lyses a culture of staphylococcus in the stage of maximum rate of growth, the observed rate of oxygen consumption is greater than can be accounted for in our opinion by the number of intact bacterial cells present.

2. It is shown that a culture lysed by the bacteriophage and containing negligible numbers of living bacteria continues to give off carbon dioxide and take up oxygen for a period of several hours.

3. A brief study of the effect on the respiration curves of varying the concentration of bacteriophage, the rate of growth, and the concentration of bacteria is presented.

CONCLUSIONS

Either the bacteriophage itself, or some product of the action of bacteriophage on or in combination with bacteria, respire.

The lysis of bacteria by the bacteriophage is not a process of simple autolysis.

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DISSOCIATION IN AN ENCAPSULATED STAPHYLOCOCCUS

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Received for publication August 20, 1930

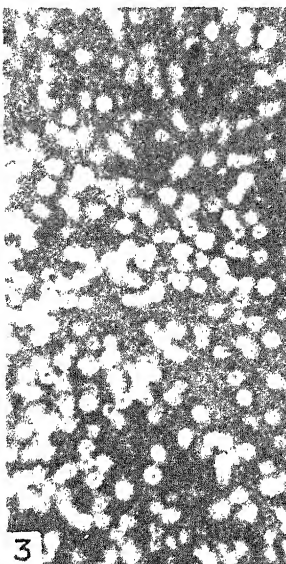
The organism to be described was isolated from an autopsy at the Johns Hopkins Hospital in April, 1929. The case was that of a young colored man with acute ulcerative gonococcal endocarditis. A typical *Staphylococcus aureus* was isolated from the heart blood and an encapsulated strain from the pericardial and the peritoneal fluids, despite the fact that there was no gross or microscopic peritonitis or pericarditis. Bacterial stains of the pericardium showed no organisms, and sections of the heart valve showed only Gram-negative diplococci.

The organisms are fairly large Gram-positive cocci. In broth (fig. 1) culture they occur most regularly singly, in pairs and occasionally in very small clusters, large groups being rarely seen except from solid media (fig. 2). They are surrounded by a well defined and extensive capsule which may include two organisms. The cocci and surrounding capsule measure 3 microns. Owing to the fact that the capsules are very soft and viscid and spread over the surface of the slide, retaining no definite outline in stained preparations, they are best observed when suspended in India ink (fig. 3).

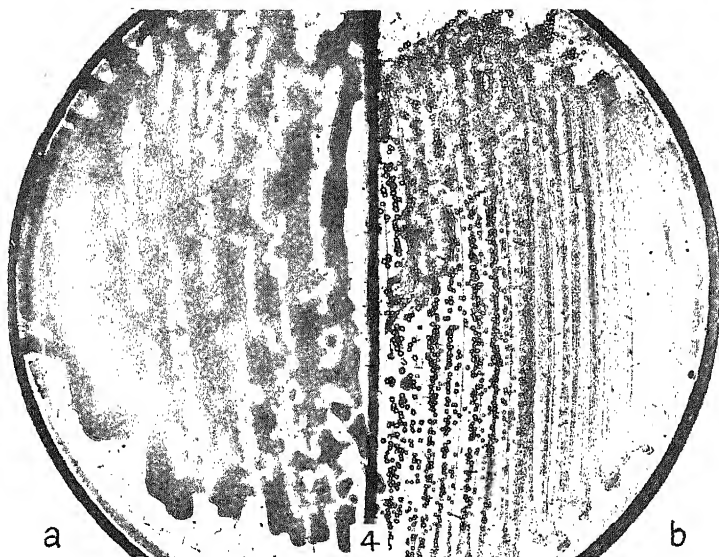
The organisms grow well on all laboratory media. On blood agar, as well as plain infusion agar, the colonies are characteristic of those of an encapsulated organism. They are large, confluent and well elevated above the agar, with a smooth glistening surface and entire edge (fig. 4a). The colonies are more translucent than those of a typical staphylococcus and are very mucoid and tenacious, adhering to the needle. On blood agar, the surface colonies

1

2



3



a

4

b

FIG. 1. GRAM STAIN OF BROTH CULTURE. $\times 1000$

FIG. 2. GRAM STAIN OF AGAR CULTURE. $\times 1000$

FIG. 3. MOIST INDIA INK PREPARATION OF S STRAIN SHOWING CAPSULES

FIG. 4. INFUSION AGAR STREAK PLATE AFTER INCUBATION FOR TWENTY-FOUR HOURS

show Beta hemolysis, while the deep colonies, which are large, bi-convex and opaque, are non-hemolytic. The growth on an infusion agar slant, on Loeffler's coagulated serum slant and on potato is heavy, spreading, smooth and glistening and, on the latter two, shows a brilliant orange color.

There is a heavy growth in infusion broth after twenty-four hours incubation, with the production of a wide yellowish-white ring at the surface of the media which in old cultures becomes bright orange in color. An abundant and very viscid sediment is formed and the culture as a whole is very thick and mucoid.

On gelatin the organism produces saccate liquefaction. In milk plus brom cresol purple the reaction is alkaline with the production of a soft curd and slowly progressive peptonization. Indol is not formed but nitrates are reduced to nitrites. Glucose, maltose, sucrose, levulose, galactose and inulin are fermented while lactose, mannitol, xylose, starch, raffinose, salicin, dulcitol, dextrin and rhamnose are not.

One month after the isolation of the encapsulated strain, small, white, opaque secondary colonies appeared on the surface of the infusion agar slants and Loeffler's slants kept at ice-box and incubator temperature. When these cultures are plated out, two types of colonies are observed. The first type is identical with those of the encapsulated strain. This strain has been called the S strain (fig. 4a). The second type is a small, round, opaque, discrete, morphologically typical staphylococcus colony (fig. 4b). No capsules can be demonstrated from these colonies or on any sub-cultures from this strain, which has been called the R strain. Dissociation into R and S forms takes place in all cultures, broth, agar slant, Loeffler's slants and milk which are from fifteen to twenty days old, but more readily in broth culture than on solid media, and at incubator temperature rather than at ice-box temperature.

Morphologically and culturally the R type organisms are identical with the S strain, with the exception of those properties which depend upon the presence of a capsule. They also occur most regularly singly, in pairs and in very small clusters and no large groups are seen except from solid media. They differ from

the S form in their opacity, and soft cheese-like consistency. On the other hand, they differ from a stock strain of *Staphylococcus aureus* in that occasionally, when injected into guinea pigs, they revert to the S form.

The encapsulated S strain is highly virulent for guinea pigs, 0.025 cc. of a twenty-four-hour broth culture injected intraperitoneally killing within twenty-four hours; while 5 cc. of the R strain produces no effect. The stock S strain used in determining the virulence is one which has been kept at ice-box temperature for a year and subcultured monthly, at each subculture, plates having been made to insure the purity of the strain and to eliminate the presence of any of the unencapsulated organisms which might have been present due to dissociation. This strain has never been passed through guinea pigs. The stock R strain used is one which has also been kept at ice-box temperature since dissociation first occurred. It has been subcultured monthly, plates having been made at each subculture and no encapsulated organisms have ever appeared. This strain also has never been passed through animals.

Large quantities of this R strain, 8 to 10 cc., occasionally kill guinea pigs. If death occurs within twenty-four hours, cultures of the heart blood and the peritoneal fluid contain only unencapsulated staphylococci in pure culture. If death occurs after forty-eight hours or if pigs which have not died in twenty-four hours are killed, cultures of the peritoneal fluid and the heart blood show both R and S forms. Control plates have been made of the R culture injected and no encapsulated organisms found. The virulence of the encapsulated strain as obtained from R by animal passage is equal to that of the original encapsulated strain.

SUMMARY

A strain of encapsulated *Staphylococcus aureus* has been described. This strain, which is highly virulent for guinea pigs, dissociates into an unencapsulated relatively avirulent strain. Reversion of the unencapsulated to encapsulated form may be brought about by passage through a guinea pig. Further work is being done in this laboratory on the antigenic and surface properties of these two strains.

A BACTERIOPHAGE FOR *B. ANTHRACIS*

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Received for publication September 13, 1930

There occur in the literature of bacteriophagy several descriptions, such as that by Pesch (1924), of pseudolytic reactions with the anthrax bacillus, but to the writer's knowledge no true bacteriophage active against this organism has ever been obtained. It is the object of this paper to report some brief studies of a lytic filtrate which, in all of its manifestations, displays the characteristics of the bacteriophage as we know it.

The following anthrax cultures were used in the investigation:

Strasbourg—obtained from Professor L. F. Rettger.

Thomas—freshly isolated from a case of malignant pustule in a tannery employee at Peabody, Mass., and obtained through the courtesy of Dr. J. E. Sullivan.

American Type Culture strains Nos. 7, 8, 9, 10, 240, 241, 242, 937, 938, 4229, 4230. Atypical Cultures Nos. 10 and 242.

All cultures were grown in alkaline extract broth of pH 7.8, or on nutrient extract agar of pH 7.0, and all were incubated at 30°C. L_8 and L_5 Chamberland candles were used for filtrations.

As a source of bacteriophage, there was used crude sewage, filtered through paper only and enriched in 100 cc. quantities with the proper amount of beef extract and peptone. To this mixture was added the growth from an eighteen-hour agar slant culture of the Strasbourg strain,—the strain which was routinely used throughout these experiments. After overnight incubation the mixed culture was filtered and the usual process of alternate feeding and filtration was pursued. One-tenth of a cubic centimeter of the third-passage filtrate exhibited some ability to clear broth cultures, and, on agar plates heavily seeded with culture,

formed numerous tiny plaques of partial lysis. After ten passages the filtrate had a titer of 2×10^{-9} as determined by plaque counts, and caused complete lysis of broth cultures in three to four hours.

The lytic principle thus obtained was highly active against the Thomas strain of *B. anthracis* as well as against all of the American

TABLE 1
Characteristics of anthrax strains used

STRAIN	MOTILITY	GELATIN LIQUEFAC- TION	FINE-TREE GROWTH	PATHOGENICITY FOR MICE*	SUSCEPTIBILITY TO ANTHRAX BACTE- RIOPHAGE	SUSCEPTIBILITY TO ATYPICAL ANTHRAX BACTERIOPHAGE
Strasbourg.....	-	-	+	+	+	-
Thomas.....	-	-	+	+	+	-
A. T. C. C. 7.....	+	-	+	-	-	-
A. T. C. C. 8.....	-	-	+	-	+	-
A. T. C. C. 9.....	-	-	+	-	+	-
A. T. C. C. 10.....	-	-	+	+	+	-
A. T. C. C. 240.....	-	-	+	+	+	-
A. T. C. C. 241.....	-	-	+	-	+	-
A. T. C. C. 242.....	-	-	+	+	+	-
A. T. C. C. 937.....	-	-	+	+	+	-
A. T. C. C. 938.....	-	-	+	+	+	-
A. T. C. C. 4229.....	-	-	+	+	+	-
A. T. C. C. 4230.....	+	-	+	-	-	-
A. T. C. C. atypical 10.....	+	+	-	-	-	+
A. T. C. C. atypical 242.....	+	+	-	-	-	+

* The mice were injected at the root of the tail with 0.1 and 0.2 cc. amounts of eighteen-hour broth cultures. A strain was considered non-pathogenic if mice failed to succumb to it within five days.

Type Culture strains with the exceptions of nos. 7 and 4230. These two strains, however, showed active motility in hanging-drop preparations, so that their authenticity is open to question.

It seems pertinent to include here the following observations. Before the complete series of Type Culture strains had been obtained, the bacteriophage was tried against two cultures which had come to us through two other laboratories and were supposed

to be strains 10 and 242. The two strains were completely resistant, and as a result another bacteriophage active against them was obtained by the method described above. This second lytic principle, however, had no effect on any of the other thirteen cultures of *B. anthracis*. A study of the two strains showed that the organisms were motile and liquefied gelatin rapidly, while 0.2 cc. amounts of twenty-four-hour broth cultures failed to kill mice in five days. In these respects the cultures were similar to *B. cereus*. The same strains obtained from the Type Culture Collection were typical *B. anthracis*. Whether the two atypical strains were contaminants or had undergone an extreme dissociation is open to question, but the fact that strains 7 and 4230 also showed motility when obtained directly from the Collection suggests that the latter possibility should receive consideration. Nungester (1929) has described the dissociation of *B. anthracis* in detail, but none of his variants were motile. Haag (1927), however, believes that motility is possible under some circumstances. Incidentally, it may be stated that the bacteriophage for the atypical strains 10 and 242 was active against *B. mycoides* as well as against an atypical culture of *B. mesentericus*. The true anthrax bacteriophage was active against the latter form also. *B. cereus* was susceptible to neither principle.

THERMOLABILITY OF THE ANTHRAX BACTERIOPHAGE

In contrast to many other lytic filtrates which can, as d'Herelle (1926) has shown, withstand temperatures up to 72° for half an hour and more, this particular principle is killed or inactivated by heating at 60° for ten minutes, although it survives 55°. On numerous occasions such treatment has been followed by as many as six serial passages against sensitive cells without the appearance of any regeneration in lytic power as evidenced by action in broth or by plaque formation.

SECONDARY GROWTH AND RESISTANCE TO LYTIC ACTION

No secondary growth has been observed in bacteriophage suspensions filtered through L₃ or L₅ Chamberland candles even

when these filtrates have stood at room temperature for over three months. In broth cultures which have undergone apparently complete lysis, secondary growth may appear. Such growth, however, seems to bear a definite relation to the number of organisms present in the original suspension as is shown by the following experiment, typical of numerous others that have been made.

Duplicate dilutions of organisms were made by adding 0.5 cc. of an eighteen-hour broth culture of *B. anthracis* to 4.5 cc. of broth and making 0.5 cc. transfers through ten tubes. One-tenth cubic centimeter of potent bacteriophage was then added to each tube of one series, while the other series served as a control. In the following table the numbers indicate the days on which turbidity was first observed. At the end of three weeks no further change was seen.

TABLE 2

	DILUTION									
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
<i>B. anthracis</i>	1	1	1	1	1	1	1	1	—	—
<i>B. anthracis</i> + Bp.....	1	6	—	—	—	—	—	—	—	—

In another experiment the dilutions were divided into four equal parts, each of which was added to a tube of broth. One-tenth cubic centimeter of bacteriophage was added to all of the tubes except the controls. Three of the portions from the 10^{-1} dilution containing bacteriophage showed secondary growth after one day. The fourth portion became turbid on the tenth day, but all of the other tubes were clear at the end of three weeks. The controls through high dilutions showed growth after one day.

These results suggest that this particular strain of *B. anthracis* may contain a very small percentage of cells resistant to lytic action, and that it is from these that secondary growth develops.

When secondary growth is subcultured in series on agar slants, the appearance in eighteen hours does not suggest the presence of any lytic agent. After further incubation, however, the culture tends to become glassy, and in these glassy areas further

growth develops. This behavior has been observed through a series of eight agar-slant cultures and, from the eighth, a potent bacteriophage was recovered, as was to be expected.

Such secondary growth produces abundant spores. If these spores are heated at 80° for ten minutes to inactivate the bacteriophage and to kill the vegetative forms, the resulting growth on agar is similar to that of normal cultures, but the organisms are resistant to the lytic action. One of these resistant strains has been carried through four cycles in which spores have been allowed to develop on agar, have undergone heating at 80° for ten minutes, and have then been allowed to germinate in broth. At the end of this series the strain had apparently retained its complete resistance to lysis. It is of interest that such resistance is carried through the spore stage and repeated heating, which indicates a rather deep-seated change in the characteristics of the strain. There seems to be no marked relationship between this resistance to bacteriophage action and virulence for mice as 0.1 cc. amounts of 18-hour broth cultures of either the susceptible or the resistant strain were able to kill in from twenty to thirty hours. The strains recovered from the mice at autopsy had retained their specific behavior to the lytic principle.

The value of the bacteriophage in immunization against anthrax as well as in therapy is under investigation.

THE VALUE OF SEWAGE AS A SOURCE OF LYTIC PRINCIPLES

Numerous investigators have made use of sewage in obtaining bacteriophages. However, in view of the success which workers in this laboratory have had in utilizing this source for lytic filtrates, it may be well to reemphasize its value for such a purpose. Aside from bacteriophages for such common organisms as *B. coli*, principles have been secured active against the following species: *C. diphtheriae*, *Bact. pneumoniae*, *B. megatherium*, *B. petasites*, *B. tumescens*, *B. anthracis*, *B. mycoides*, and several other spore-forming bacilli of doubtful classification.

SUMMARY

A bacteriophage highly potent for *B. anthracis* has been obtained from sewage. All of the typical anthrax strains studied, eleven in number, were susceptible to its action.

It is possible to develop resistant strains of *B. anthracis* in which the resistance is carried through the spore stage and maintained through repeated pasteurizations.

The occurrence of secondary growth with the strain of *B. anthracis* used in these experiments, at least, seems to be dependent upon the number of organisms subjected to the lytic principle.

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ISOLATION AND STUDY OF AN APPARENTLY WIDE-SPREAD CELLULOSE-FERMENTING ANAEROBE, CL. CELLULOSOLVENS (N. SP.)¹

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Received for publication September 15, 1930

The decomposition of cellulose by microbiological agents has for many years attracted considerable attention. This is not at all surprising, for cellulose occurs in enormous quantities in nature and is of great economic importance. Furthermore, its extreme resistance to most chemical and biological influences makes it all the more interesting.

The earlier investigators who attacked the problems of cellulose decomposition by bacteria dealt with crude cultures only, and were inclined to the belief that the process was essentially an anaerobic one. However, in 1904 Van Iterson observed that cellulose could be destroyed under aerobic conditions, and described the organisms concerned. As a result, research has, for the most part, been concentrated on this phase of the problem, and there have been isolated many aerobic bacteria, facultative anaerobes and fungi, which are active in the process. Among the workers in this field are Kellerman and McBeth (1912) and Bradley and Rettger (1927). Thaysen and Bunker have reviewed the subject from many aspects in their "Microbiology of Celluloses, Hemicelluloses, Pectin and Gums" (1927).

The anaerobic bacteria, on the other hand, have received relatively little attention, at least in so far as the isolation of pure cultures is concerned. This is probably due to the fact that, while easy to demonstrate in mixed cultures, they are extremely exacting in their growth requirements and are very difficult to obtain

¹ This paper is based upon the senior author's Doctorate Thesis deposited in the Yale University Library.

by themselves. As far as the writers are aware, only three investigators have made claims of isolation of pure strains. First among these was Omeliansky (1902), who published several papers in which were described two cellulose fermenting anaerobes, *B. fossicularum* and *B. methanicus*, obtained from river mud and horse feces by growth in an inorganic salt medium containing cellulose. Both were thin rods which formed spherical terminal spores. Cellulose was essential to these organisms and was, indeed, the only carbohydrate which they could attack. Carbon dioxide and various organic acids were formed while, in addition, *B. fossicularum* produced hydrogen, and *B. methanicus*, methane. Omeliansky was unable to secure colony formation on any solid medium and had to resort to repeated heatings and dilutions to secure his isolations. Kellerman and McBeth believed that these two organisms were not in reality pure cultures, since they were able to obtain several contaminating forms from them, including a cellulose-destroying facultative anaerobe.

In 1923 Khouvine described another anaerobe, *B. cellulosaedissolvans*, which was strictly specific for cellulose and dependent upon it for growth. This organism was similar to Omeliansky's in its inability to attack sugars and to grow on solid media, but it could be distinguished from the other by the formation of oval spores. Isolation was secured by repeated heating, diluting and growing in a special fecal extract medium containing cellulose. By her technique Khouvine was able to demonstrate the presence of the organism in the excreta of man and of the herbivora, and in numerous soils. The products of fermentation were found to be carbon dioxide, hydrogen, organic acids and ethyl alcohol.

The third report deals with a description of *B. cellulosa-fermentans* isolated by Werner in 1926 from the intestines of rose beetle larvae. Morphologically it is similar to Khouvine's organism, but the oval spore is slightly smaller. In Werner's hands the isolation methods used by the preceding workers were unsuccessful; he adopted the following technique. An enriched culture of the organism was developed in the usual way in Omeliansky's medium. A bit of the decomposing paper was next streaked thoroughly over the surface of a nutrient agar plate which was

then incubated for twenty-four hours. At the end of this time all areas of visible growth were cut out with a sterile knife and the same plates incubated further. If no new growth became apparent, the surface of the agar was wiped off with a bit of sterile cotton in order to gather up the dormant spores of the desired organism, and the cotton planted in a tube of medium. Disintegration of the filter paper followed in from four to ten days. The cultures were assumed to be pure when no growth occurred on ordinary nutrient agar, incubated both aerobically and anaerobically for several days, and when microscopic observation showed no contaminating forms. Werner could obtain growth only in the presence of cellulose, as apparently no other carbohydrates were utilized. The only solid medium on which growth took place was the cellulose agar of Kellerman and McBeth, and on this the organism developed only occasionally, and in contact with fibers. Gas formation was observed, but the fermentation products were not investigated.

From the foregoing brief summary it is apparent that the isolation of cellulose-fermenting anaerobes is fraught with difficulties. Not the least of these is the uncertainty of knowing whether or not the cultures are pure, for failing colony formation or single cell isolation, the present-day methods of obtaining pure strains are open to criticisms.

This paper deals with the isolation, growth upon solid medium, and study of an anaerobic organism which is morphologically similar to those described by Omeliansky.

Media

1. Omeliansky's medium

Ammonium sulphate or peptone.....	1.0 gram
Di-potassium phosphate.....	1.0 gram
Magnesium sulphate.....	0.5 gram
Sodium chloride.....	trace
Calcium carbonate.....	excess
Water.....	1000cc.

2. Fecal extract medium

Peptone.....	1.0 gram
Di-potassium phosphate.....	1.0 gram
Sodium chloride.....	1.0 gram

Calcium carbonate.....	excess
Fecal extract.....	250 cc.
Water.....	750 cc.

The fecal extract was prepared by extracting horse feces with ten parts of water, filtering through paper, autoclaving, and passing through a Berkefeld candle.

3. Beef infusion broth

Beef infusion.....	1000 cc.
Peptone.....	5.0 grams
Di-potassium phosphate.....	1.0 gram
pH.....	7.0

In all of the above media cellulose was added in the form of a strip of filter paper placed in each tube.

ANAEROBIC METHODS

All culture media were autoclaved and cooled immediately before inoculation. Anaerobic conditions were obtained, as a rule, by placing the cultures in jars, exhausting with a vacuum pump until the liquid boiled (or, in the case of agar media, to a constant pressure), admitting carbon dioxide and re-exhausting several times, and finally sealing off the jar after the last exhaustion. Incubation was always at 37°C.

THE PREVALENCE OF OBLIGATE ANAEROBIC CELLULOSE-FERMENTING BACTERIA

The presence of spore-bearing cellulose-fermenting bacteria in soils, muds, feces, and in fact wherever cellulose is undergoing decomposition, is easily demonstrable. Dügelli (1921) found the following numbers per gram of soil: garden, vineyard, and meadow, 367; field, 350; and marsh, 1.1. In the present investigation samples from numerous sources were collected in sterile tubes. In the case of the soils, the surface was first scraped away and then material taken to a depth of three inches. The fecal specimens were always from fresh excreta.

Ten-gram portions of a well-mixed sample were placed in dilution bottles containing enough sterile water to bring the final volume to 100 cc. and thoroughly shaken. From these bottles, 10 cc. transfers were made to bottles containing 90 cc. of water,

and so on through several dilutions. One cubic centimeter amounts of the different dilutions were then added to tubes containing about 15 cc. of Omeliansky's medium with peptone. These tubes were heated at 80°C. for ten minutes, cooled, and placed under anaerobic conditions. The presence of spore-forming cellulose fermenters was shown by disintegration of the filter paper, accompanied by gas formation, in from five to ten days.

Numerous samples of soils and feces, besides those listed, were examined, and positive results almost invariably obtained. Mud from the bottom of a salt water pond was particularly active. Feces from four dogs seemed to be devoid of cellulose fermenting

TABLE 1
Anaerobic spore-forming cellulose-fermenting organisms from various sources

SOURCE	SMALLEST AMOUNTS OF NORMAL SAMPLE TO ATTACK CELLULOSE	EQUIVALENT OF DRY SAMPLE	ORGANISMS PER GRAM DRY SAMPLE
	<i>grams</i>	<i>grams</i>	
1. Lawn.....	0.01	0.009	110
2. Marsh.....	0.01	0.004	250
3. Potato field.....	0.01	0.009	110
4. Pine woods.....	No attack		
5. Rich garden soil.....	0.001	0.0009	1,100
6. Cow feces.....	0.01	0.002	500
7. Human feces.....	0.01	0.003	333
8. Rabbit feces.....	0.05	0.03	33
9. Dog feces.....	No attack		

anaerobes, a fact rather to be expected, since dogs are essentially carnivorous.

This method, at best, is very inaccurate and gives only a rough idea of the numbers of organisms present. There were, undoubtedly, many more than the determinations showed. The limited number of samples taken does not make it permissible to generalize too broadly, but one may conclude from these findings that cellulose-fermenting anaerobic spore-formers are widely distributed in nature, and that they probably play a more important rôle in nature's economy than is usually attributed to them. A further study of this rôle should reveal some interesting facts,

particularly in so far as the digestion of cellulose in the intestine is concerned.

ISOLATION

In all of the cultures in which active decomposition of cellulose was observed an organism was present which morphologically resembled those described by Omeliansky. The rods appeared slender and often slightly curved; they were about 0.5 micron in diameter and from 2 to 6 micra long. A spherical terminal spore varying from 1.0 to 1.5 micra in diameter was often seen either attached to the rod or free. It is this organism or one morphologically similar to it which has been described by different workers in the field of cellulose fermentation.

The present attempts to effect its isolation were made from horse feces by the preliminary enrichment culture method. The medium described by Khouvine was employed, except that horse, instead of human, fecal extract was added. After heating in this medium for fifteen minutes at 80°C. and incubating in a vacuum the forms that persisted were few in number, with the typical cellulose-fermenting type predominating.

Both Omeliansky and Khouvine seemed able to eliminate the contaminating forms by repeated transfers and heatings. This method, however, was not successful in Werner's work, nor was it effective in the present investigation. In some instances, where all contaminants had apparently been eliminated as far as microscopical and cultural studies showed, no further growth of the cellulose organism could be demonstrated.

The dilution method was tried, but the results were equally unsatisfactory. The last tube which revealed cellulose decomposition always contained a contaminant.

Attempts were next made to produce colonies on various solid media. Meat extract and meat infusion agar with and without glucose, maltose and soluble starch, and tomato agar, casein digest agar and silica gel were used. The addition of precipitated cellulose prepared by the method described by Kellerman and McBeth was tried in several of the above media. In no case was colony formation observed.

Numerous methods of obtaining anaerobic conditions were also tried. The exhaustion and replacement system already described, Marino's cupped plates (1907), growth with non-spore forming bacteria as recommended by Sturges and Rettger (1919), and deep agar shake tubes were alike unsuccessful.

In a few instances colonies of organisms resembling the one desired were noticed, but from these no growth or cellulose decomposition could be obtained. This observation tallies with that of Choukevitch (1911) who, in studying the intestinal flora of the horse, was able to isolate an organism which was morphologically similar to Omeliansky's. He, also, failed to make it attack cellulose in pure culture.

At this point a device used originally by Winogradsky (1890) and later by Werner was tried in a modified form. Nutrient agar plates were streaked with a small bit of washed paper from an almost pure fermenting culture of the cellulose organism. The plates were incubated aerobically for two days at 37°C. Colonies of contaminants developed along the first parts of the streak, but the more thinly streaked areas showed no growth. Small portions of agar were cut out from these clear parts with a sterile spatula and transferred to tubes of fecal extract medium and to Omeliansky's solution. In some of these tubes decomposition of the paper was apparent in from four to ten days, but on examination there was always found an oval spore-producing contaminant.

Since the cellulose-attacking type was microscopically the most abundant form in the culture with which the plate had been streaked, it seemed highly probable that its spores predominated along the parts of the streaked agar which showed no growth, and that, therefore, some of these spores must have been transferred to tubes of medium where they failed to develop. In these tubes the spores remained dormant unless an occasional contaminant served to make conditions favorable for their germination. Such associative influence could easily explain the results obtained in this experiment as well in many of the previous unsuccessful attempts to secure growth.

Acting on this suggestion, another plate was treated as described above, but in this instance the small portions of agar were

transferred to tubes which were inoculated also with a non-spore former, *Bact. aerogenes*. In most of these tubes decomposition of the paper was observed after suitable incubation. Some of them contained contaminating spore forms, but others seemed to have only the cellulose fermenting organism plus *Bact. aerogenes*. The last-mentioned tubes were heated at 80°C. for fifteen minutes to kill all vegetative cells, and purity tests were made by inoculating various media and incubating both aerobically and anaerobically. No evidences of contamination could be seen. The cultures were considered pure, therefore, and from them inoculations were made into tubes of fecal extract medium, some of which also received *Bact. aerogenes*. The tubes containing the

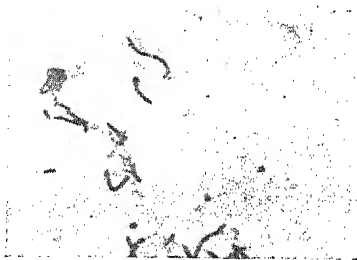


FIG. 1. PHOTOMICROGRAPH SHOWING VEGETATIVE CELLS, A SPORANGIUM AND SPHERICAL SPORES. 1000X

associated organism consistently showed cellulose decomposition; the others did not.

By using the same principle (association with *Bact. aerogenes*) and the dilution method a second isolation was effected.

The failure to obtain growth in pure culture without the influence of some associated organism (*Bact. coli*, *Proteus vulgaris*, and an unidentified spore-forming anaerobe were likewise effective) seemed to indicate either that the food requirements of the cellulose organism were not satisfied by any of the media used or that bacterial association is absolutely essential in its development. The former hypothesis seemed to be the plausible one.

In further efforts to find a suitable medium, cellulose meat infusion was tried and found to be very satisfactory. It is quite useless so long as any contaminating forms are present, since these as

a rule develop so luxuriantly as to overgrow the cellulose organism. In this meat infusion medium, pure culture decomposition of the filter paper was observed in from three to five days. The body of the liquid became only slightly turbid, indicating that the organism grows abundantly only in contact with the cellulose fibers. Gas bubbles were evolved slowly, often forming blisters in the paper. In the course of several days after the fermentation set in the paper disintegrated and fell to the bottom of the tube, and the reaction subsided, probably interrupted by the increased H-ion concentration. The pH at this time was about 5.6.

MORPHOLOGY

Vegetative cells in liquid media are about 0.5 micron in diameter and from 2 to 6 micra in length, sometimes slightly curved and often occurring in pairs. Chains of more than two cells are not seen.

The spores form as dark-staining granules in the end of the rod, usually when it has reached a length of 2.5 to 3.5 micra. This granule swells to a diameter of 1.0 to 1.5 micra. The free spores are almost perfect spheres.

STAINING REACTIONS

The organism stains readily with all of the common dyes. The Gram stain is rather uncertain. As a rule the cells seem to be Gram-negative.

MOTILITY

The organism is non-motile when viewed by the usual hanging drop method.

FERMENTATION REACTIONS

It has been observed by all workers in the field of anaerobic cellulose fermentation that their organisms have been highly specific in their carbohydrate requirements. Indeed, there appears to be no mention in the literature of attack on any substance except cellulose.

In the present investigation, such common carbohydrates as glucose, maltose, and soluble starch were occasionally used in efforts to find some soluble energy source to replace the filter paper, as this in its natural form or as precipitated cellulose is very inconvenient for incorporation in agar media. In none of these attempts could growth be uniformly obtained, although at times and after prolonged periods of incubation there were some evidences of development. With a pure culture available, and a medium which favored development, the study of the fermentation characteristics of the organism was begun.

Valley (1929) has shown that ordinary Dunham inverted vial fermentation tubes are quite adequate for the study of fermentation by anaerobes, provided 0.1 per cent of cysteine hydrochloride is added to the medium. The writers have found that the organism under investigation develops very nicely in such tubes, even when the cysteine is omitted. In fact, when inoculations were made into these tubes and into ordinary culture tubes incubated *in vacuo*, attack on the cellulose occurred in both after the same period of incubation. These simple fermentation tubes were employed, therefore, in the following study.

The basic medium used was the standard meat infusion broth to which one per cent of the various carbohydrates was added. The reaction was adjusted to pH 7.0 to 7.2, and after autoclaving at 15 pounds for ten minutes was tested again to make certain that no change had occurred. With the pentoses, arabinose and xylose, a change in the color of the medium to a deeper yellow accompanied by a drop in reaction to pH 6.4 was observed, indicating some degree of decomposition. Alkali was added to bring the reaction back to the original level.

All of the carbohydrates were tubed in quadruplicate; two tubes of the sugar medium, with and two without strips of cellulose, constituted a set. Controls of plain meat infusion, with and without cellulose, were used.

The inoculum was 0.1 cc. of a seven-day culture in cellulose meat infusion medium. Care was taken to transfer no cellulose fibers in the process of inoculation.

The following substances were employed:

<i>Pentoses</i>	<i>Hexoses</i>
Arabinose	Glucose
Xylose	Levulose
	Mannose
<i>Trisaccharides</i>	<i>Polysaccharides</i>
Melizitose	Soluble starch
Raffinose	Dextrin
	Inulin
	Cellulose
<i>Alcohols</i>	<i>Disaccharides</i>
Adonitol	Lactose
Dulcitol	Maltose
Erithrytol	
Glycerol	<i>Glucosides</i>
Inositol	Amygdalin
Mannitol	Salicin
Sorbitol	
<i>Gums</i>	
Gum Arabic	

The data presented in table 2 show that this organism is highly restricted in its ability to utilize carbohydrates. At times, an occasional tube containing a test substance alone showed some evidences of fermentation. This was infrequent, however, and was probably the result of a partial hydrolysis during autoclaving. The slight but consistent fermentation of soluble starch may also be attributed to this cause.

Furthermore, these results confirm the reports of other workers that glucose is not utilized by the anaerobic cellulose fermenting organisms. This is all the more interesting inasmuch as it is generally believed that, if bacteria are able to decompose any sugar, they can attack glucose.

It is also significant that the cellulose is readily attacked in the presence of all of the substances used except arabinose and xylose. Glycerol seems to exert a complete inhibition upon the development of the organism, and lactose a partial restraint. Even dextrin, which is readily available as an energy source, does not prevent an attack upon the more complex cellulose.

Of more practical value from the point of view of a study of the anaerobe is its ability to ferment dextrin. Cysteine meat infusion agar containing this substance served as an excellent medium on which to secure colony formation. After three days' incubation

TABLE 2

Showing the action of the isolated organism on carbohydrates, alcohols and glucosides

CARBOHYDRATES, ETC.	CARBOHYDRATES, ETC., ALONE			CARBOHYDRATES, ETC., AND CELLULOSE		
	Gas observed	Final per cent gas	Final pH	Gas observed	Final per cent gas	Cellulose decom- position
	<i>days</i>			<i>days</i>		
Control.....	—	—	7.2	3	65	+
Arabinose.....	5	30	6.2	4	45	—
Xylose.....	6	20	5.8	4	40	—
Glucose.....	—	—	7.2	3	60	+
Levulose.....	—	—	7.2	5	60	+
Mannose.....	—	—	7.2	3	55	+
Lactose.....	—	—	7.2	—	—	—
Maltose.....	—	—	7.2	5	55	+
Sucrose.....	—	—	7.2	3	60	+
Melizitose.....	—	—	7.2	5	50	+
Raffinose.....	—	—	7.2	4	60	+
Soluble starch.....	4	2	6.9	3	55	+
Dextrin.....	4	25	6.5	3	65	+
Inulin.....	—	—	7.2	4	60	+
Cellulose.....	3	65	5.6	4	65	+
Salicin.....	—	—	7.2	4	40	+
Amygdalin.....	—	—	7.2	4	40	+
Adonotol.....	—	—	7.2	4	50	+
Dulcitol.....	—	—	7.2	5	55	+
Erythritol.....	—	—	7.2	5	50	+
Glycerol.....	—	—	7.2	—	—	—
Inositol.....	—	—	7.2	4	50	+
Mannitol.....	—	—	7.2	3	60	+
Sorbitol.....	—	—	7.2	4	55	+
Gum arabic.....	—	—	7.2	4	60	+

in vacuo or under hydrogen, small, discrete colonies appeared. These seldom grew to more than 0.5 mm. in diameter, even after prolonged incubation. They were round and had a transparent, dew-drop appearance. The body of the colony was finely granular in structure, with a smooth edge. Inoculations from these

colonies always gave growth in cellulose meat infusion in from two to four days, and caused decomposition of the filter paper. As a rule, relatively few spores were seen in pure cultures, even after continued holding under aerobic or anaerobic conditions.

THE INFLUENCE OF VARIOUS CARBOHYDRATES UPON THE AMOUNT OF CELLULOSE DESTROYED

In the study of the fermentation characteristics of the anaerobe it was noticed that, whenever cellulose and other carbohydrates were present, the cellulose was attacked in preference to the latter, except in the cases of arabinose and xylose. In view of the fact

TABLE 3

Showing the influence of glucose, starch and dextrin upon the amount of cellulose decomposition

CARBOHYDRATES	ORIGINAL WEIGHT PAPER	AMOUNT OF PAPER DESTROYED	PER CENT OF GAS	FINAL pH
	<i>mgm.</i>	<i>mgm.</i>		
1. Cellulose only.....	85	17	45	6.4
2. Cellulose only.....	84	*	0	7.4
3. Cellulose + glucose.....	87	16	45	6.4
4. Cellulose + glucose.....	85	14	50	6.4
5. Cellulose + soluble starch.....	86	16	55	6.4
6. Cellulose + soluble starch.....	88	15	55	6.4
7. Cellulose + dextrin.....	86	11	50	6.4
8. Cellulose + dextrin.....	82	10	55	6.4
9. Cellulose (uninoculated).....	84	2	0	7.2

* No growth.

that both dextrin and, to a slight extent, soluble starch could be used without preventing the fermentation of the cellulose, it seemed of interest to determine their influence upon the amount of cellulose that is decomposed. Glucose was also included in the experiment, as its failure to be utilized is so unusual.

Weighed strips of paper were placed in fermentation tubes containing 15 cc. of meat infusion, pH 7.2. These were inoculated with 0.1 cc. of an actively fermenting cellulose meat infusion culture, care being taken to transfer no cellulose fibers. The cultures were observed daily, and at the end of eight days were taken

from the incubator and final determinations made. The filter paper used in the experiment had been dried at 105°C. to constant weight. After the fermentation in the culture tubes had subsided, the contents of each tube were filtered through a Gooch crucible similarly dried to constant weight, and washed several times alternately with one per cent hydrochloric acid, one per cent potassium hydroxide and distilled water. The crucibles with their contents were then thoroughly dried and the weight determined.

It will be seen from the figures in table 3 that the presence of dextrin inhibits to some extent the decomposition of the cellulose, probably because the dextrin is partly responsible for the lowering of the pH. On the other hand, neither soluble starch nor glucose have any effect upon the cellulose fermentation.

GAS PRODUCTION

The observation has been made by all workers in the field of anaerobic cellulose fermentation that gas is consistently produced in their cultures. Omeliansky believed that he could produce a methane as well as a hydrogen fermentation by his two supposedly pure cultures. Khouvine, however, found only hydrogen and carbon dioxide in the evolved gas, and Werner, although he failed to make analysis of the gas from his pure culture fermentation, found no methane in the product of crude cultures.

In the present experiment the apparatus described by Werner (1926) was used. This consists of two bottles connected with an inverted U tube which passes through a cotton plug in one (the overflow bottle), and through a two-hole rubber stopper in the other (the culture bottle). This tube should reach to the bottom of each bottle. Through the second hole of the rubber stopper a short glass tube passes. This is connected by a piece of soft rubber tubing about an inch long to another short glass tube plugged with cotton. The soft rubber tube is supplied with a pinchcock with which to control the flow of gas.

The cellulose is placed in the culture bottle, each bottle is filled over half full with the medium (in this case the standard meat infusion), the stopper and cotton plug are tightly fitted, and the

whole system is autoclaved. Care must be taken not to have the pinch-cock close the rubber tube, as the air must have a means of exit in the autoclave. It is advisable to cover the rubber stopper with cotton and then with paper, in order that no contaminating organisms may enter the bottle through its junction with the glass.

After autoclaving, the medium is immediately sucked up to fill the culture bottle completely so that no air can enter. When the whole system has cooled, the cotton plug in the short tube at the top of the culture bottle is withdrawn, using appropriate precautions, and the inoculum is admitted by means of a pipette.

In this experiment 1.0 cc. of an actively fermenting pure culture of the cellulose decomposing anaerobe was used as inoculum.

Active gas evolution began after three days' incubation and lasted for five days. At the end of this period slight action was still evident, but the culture was removed and analyzed.

Data on the fermentation are listed below.

Volume of culture bottle.....	240 cc.
Weight of cellulose used.....	1.178 gram
Weight of cellulose recovered.....	0.746 gram
Weight of cellulose destroyed.....	0.432 gram
Gas evolved 1st day of fermentation.....	15 cc.
Gas evolved 2nd day of fermentation.....	40 cc.
Total gas evolved in five days.....	130 cc.
Amount of normal acid as titrated with N/10 NaOH.....	6.4 cc.

The data on the gas volumes are not at all accurate for several reasons. Pressure and temperature were ignored, as was the amount of gas which was dissolved in the culture medium. Two analyses of the gas made at different stages of its evolution, in a modified Orsat apparatus described in the Bureau of Mines Bulletin No. 197, showed that of the gas about 75 per cent was hydrogen, and the remainder carbon dioxide.

SUMMARY

1. There has been isolated from horse feces a cellulose fermenting anaerobe (for which the name *Clostridium cellulosolvens* is proposed) morphologically similar to the organisms described by Omeliansky. The rods are thin, often slightly curved, and form spherical terminal spores.

2. Colony formation can be secured on a peptone beef infusion agar medium containing dextrin and cysteine. This medium is not selective and cannot be used for direct isolation, but is of value in obtaining the desired organism from enriched cultures. The best liquid medium devised for cellulose decomposition by this organism consists of a peptone beef infusion phosphate broth containing a strip of filter paper.

3. Cellulose, dextrin, arabinose and xylose alone of the materials tried are attacked. Glucose is not utilized, a fact in contradiction of the so-called theory of carbohydrate gradients. The presence of dextrin or glucose in the medium seems to have little or no effect upon the amount of cellulose decomposed.

4. As far as they have been determined, the products of cellulose decomposition by this organism are carbon dioxide, hydrogen and organic acids.

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THE BIOLOGICAL CHARACTERS OF *B. ACTINOIDES* VARIETY *MURIS*

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Received for publication September 14, 1930

The isolation of a pleomorphic encapsulated microorganism from exudate in the middle ear cavity of naturally infected albino rats has been previously reported (Nelson, 1930a). The peculiar morphological characters were deemed sufficient to identify it tentatively as *B. actinoides*. A more extended study of the organism confirmed the classification but revealed certain differences from the type species originally isolated by T. Smith (1918) from the pneumonic lungs of calves. The biological characters of the rat type are presented in detail and compared in particular instances with those of the original species derived from the calf.

GENERAL CHARACTERS OF THE ORGANISM

The present type of *B. actinoides* was first observed in exudate implanted as discrete deposits on the surface of slanted agar. Stained films of exudate from cultures which had been incubated at 37°C. for twenty-four hours often showed Gram-negative elements varying markedly in size, shape, and arrangement. The most common forms were rods and filaments which frequently displayed deeply stained, central or terminal, sporelike expansions. Large capsules of irregular shapes characteristic of *B. actinoides* always developed in the fluid at the base of secondary cultures on sealed coagulated horse serum.

Unsealed slanted agar (pH 7.6) with 1 cc. of 50 per cent bovine serum bouillon at the base was found to support an abundant growth of the organism. This medium proved to be an excellent one for routine culturing and was employed in a bacteriological

study of the flora of the tympanic cavity in middle ear disease of the albino rat. The organism remains viable on this medium for a period of about two weeks, either at room or at ice box temperature.

In practice, several loops of exudate were deposited at intervals along the slanted surface of the agar and an additional loop mixed with the fluid at the base. The presence of *B. actinoides* in the exudate was attended by a floccular growth in the fluid. The production of capsules was generally delayed until the second day. There was no visible surface growth during the first 24 hours but microscopical examination of the exudate showed the presence of highly pleomorphic rods and filaments. If the tube was agitated, dispersing the sediment over the slanted portion, small colonies appeared on the second or third day.

Serum-agar transfers from both the exudate and the fluid of pure initial cultures always reproduced the same general growth characters. With the secondary cultures, a 2 mm. loop of fluid or a bit of exudate was transferred to the serum bouillon and the tube tilted several times to wet the surface of the slant. After incubation at 37°C. for twenty-four hours, growth was visible in the fluid portion of the medium as a scanty white sediment together with very fine aggregates in suspension. The sediment was composed of larger clumps which were readily dispersed, but not broken up to any extent, on agitation. The sediment increased in bulk through about the 3rd day and finally occupied between a fifth and a fourth of the column of fluid at the base of the tube. At this time the supernatant was practically clear. There was generally an extension of the growth, probably by sedimentation, downwards between the agar and the wall of the tube. Minute colonies were visible by the second or third day on the slanted surface of the medium. Most of them showed little subsequent increase in size but a few attained diameters up to 1 mm. The colonies were usually discrete but sometimes appeared to form a continuous film.

Unstained hanging drop preparations from twenty-four-hour-old sediment showed irregularly shaped aggregates of varying size. The clumps were of two general types. One was loosely

formed and composed chiefly of tangled filaments. The other was more compact, composed chiefly of bacillary forms. A very occasional capsule was visible at this time. These were more numerous after forty-eight hours and increased in number for several days, sometimes completely masking the underlying structure of the clump. More commonly, however, they were found scattered at intervals throughout the mass, apparently with no particular localization. Capsules were also present outside the clumps, as individual elements. They varied markedly in shape and size. Some were very minute and difficult to distinguish, but more often they were voluminous with sharply outlined mar-

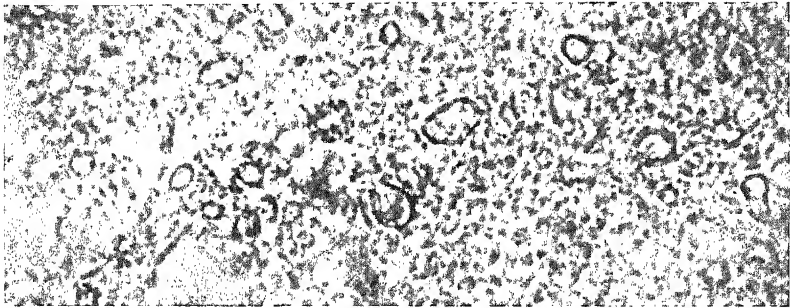


FIG. 1. PORTION OF A LARGE COMPACT AGGREGATE FROM FLUID AT THE BASE OF A SEVEN-DAY OLD SERUM-AGAR CULTURE OF *B. ACTINOIDES* VAR. *MURIS*

The film is unstained and spread between the slide and coverslip. The detailed structure of the clump is not well brought out because of its thickness. $\times 920$.

gins, at times doubtly refractive. Spherical forms were particularly numerous. Clubshaped capsules, radially arranged, which are particularly characteristic of the calf variety of *B. actinoides* were much less common. A section of an unstained clump of *B. actinoides* from the fluid of a seven-day-old culture is shown in figure 1. A number of large capsules of irregular shape are visible.

The aggregates from twenty-four- and forty-eight-hour-old sediments stained well in dried films. The individual elements were always Gram-negative. The filaments generally showed

cross walls or open spaces between small segments but in some instances they appeared as continuous threads. The filaments and rods often displayed protoplasmic irregularities, visible as beading and spherical or tubular expansions. Capsules were never seen in stained films. About the third day the clumps began to lose their staining ability, appearing as structureless, faintly stained masses in which an occasional sharply outlined filament or rod was visible. A stained clump composed largely of filaments, from a twenty-four-hour-old sediment, is shown in figure 2.

Stained films of the surface growth from forty-eight- to seventy-two-hour-old bovine serum-agar cultures showed Gram-negative

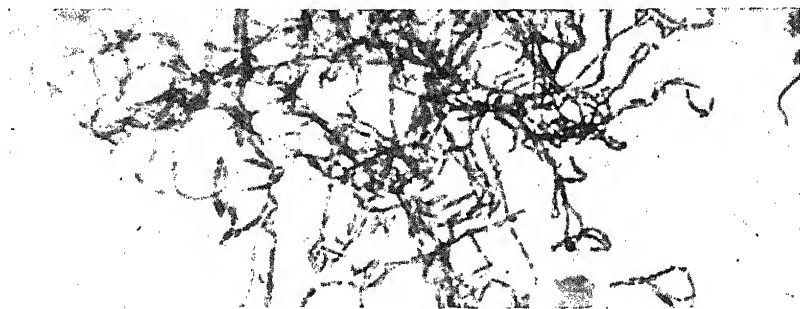


FIG. 2. MARGIN OF A LARGE FILAMENTOUS AGGREGATE FROM FLUID AT THE BASE OF A TWENTY-FOUR-HOUR-OLD CULTURE

Film is dried and stained with dilute fuchsin. $\times 1000$

elements which displayed an extreme degree of pleomorphism. Rods and filaments of varying size were the most common forms. They were rarely grouped as formed aggregates. Cellular irregularities were particularly striking. These included beading of the protoplasm; sharply outlined, deeply stained, spherical or tubular expansions; and large coccoid elements arranged singly or in chains and irregular clumps. Generally all of these forms were present in films from any one culture but no single form predominated throughout a series of cultures. Some of the filaments showed either cross walls or gaps, some appeared as continuous threads. Capsules were rarely seen in hanging drop

preparations made from the surface growth. Films made from four- to five-day-old cultures showed only an occasional sharply stained and outlined cell. Figure 3, made from a forty-eight-hour-old surface growth, dried and Gram stained, shows the various morphological elements described above.

The microscopical picture was quite different with stained films made from the early growth on the surface of unsealed coagulated horse or bovine serum. Here the predominating element was a slender Gram-negative bacillus. Long slender filaments were also present in smaller numbers. Staining was never as intense as with the previously described forms and the marked cellular

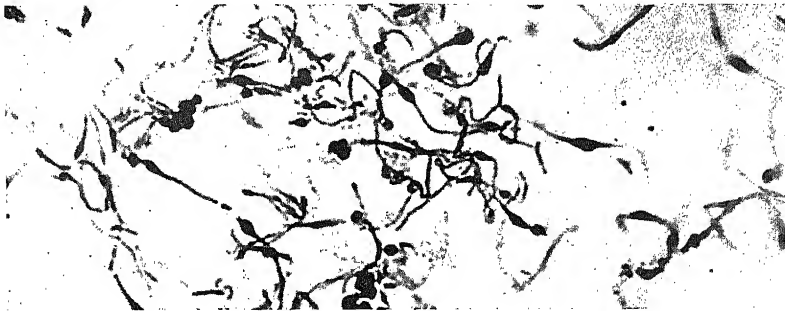


FIG. 3. SURFACE GROWTH OF THE ORGANISM FROM A FORTY-EIGHT-HOUR-OLD SERUM-AGAR CULTURE

Film is stained with dilute fuchsin. $\times 1000$

irregularities were largely lacking. Stained films always showed a few beaded and thickened cells. Transfers from the surface growth to the serum-agar medium reproduced the usual pleomorphic morphology. The microscopical character of the surface growth on coagulated serum closely resembles that of the calf type of *B. actinoides* on the same medium. In both cases bacilli which display no striking cellular irregularities predominate. Cells with largesporelike expansions are not a characteristic feature in either case. Figure 4 shows the morphological elements typical of the surface growth on coagulated bovine serum.

The capping of a medium with sealing wax had no effect on the development of the organism, irrespective of the medium em-

ployed. There was no significant variation in either the macroscopical or the microscopical characters of growth with sealed and unsealed cultures. It should be noted that the cotton plugs of agar slants for general bacteriological use at this laboratory are impregnated with paraffin. This practice retards evaporation but causes no appreciable reduction in the oxygen tension. Sealing with sealing wax prevents evaporation and also the free interchange of oxygen. These latter conditions tend to favor the development of the calf variety particularly as regards surface growth.

In the routine examination of exudate from diseased rats, mixed cultures of bacteria were often encountered but pure cultures of



FIG. 4. SURFACE GROWTH OF THE SAME STRAIN FROM A FORTY-EIGHT-HOUR-OLD CULTURE ON COAGULATED BOVINE SERUM
Stained as before. $\times 1000$

the organism were by no means uncommon. Aside from very rapidly growing microorganisms like *B. proteus* the presence of other bacteria did not prevent the development of *B. actinoides*. In the presence of *B. proteus*, which rapidly forms a diffuse growth in the fluid and a thick film over the slanted surface of the medium, the slow growing *B. actinoides* was largely suppressed. With a particularly abundant development of micrococci in the fluid portion of the medium the aggregates of the organism were generally small and few in number. The production of capsules was not inhibited. Stained films from the implanted exudate often showed the typical pleomorphic cells of *B. actinoides* together with other bacteria.

Cultivation of *B. actinoides* on other media was attempted. Unenriched nutrient agar and bouillon failed to support growth. Due presumably to the simultaneous carriage of serum the aggregates transferred upon inoculation sometimes increased in size. Secondary transfers to unenriched medium showed no development. Agar enriched with 1 cc. of defibrinated horse or bovine blood gave a somewhat scanty but otherwise typical growth of the organism. The aggregates at the base showed a good distribution of capsules. Growth in whole blood was not accompanied by hemolysis. There was no development, either at the base or on the slant, with agar enriched with 1 cc. of well washed horse or bovine erythrocytes diluted 1:5 with saline. Agar enriched with 1 cc. of milk whey (bovine) filtered through a medium Berkefeld candle gave a scanty granular growth at the base with a few colonies on the slanted surface. At times the sediment was pigmented, black or brown in color. Capsules were very rarely observed. Agar enriched with 1 cc. of ascitic fluid gave a somewhat similar growth. The sediment was scanty and the surface growth sparse. The sediment was not pigmented and showed only an occasional capsule.

Coagulated bovine and horse serum with 1 cc. of either 50 per cent bovine serum bouillon or 10 per cent calf serum water at the base supported a good growth of the organism. Large and small aggregates were formed in the fluid portion of the medium. Capsules were regularly produced but were less numerous in the presence of the calf serum water. Growth on the slanted portion of the medium was somewhat more abundant than on plain agar enriched with serum. In general, coagulated serum is not as satisfactory as plain agar enriched with serum for the cultivation of the rat type of *B. actinoides*. With the calf type coagulated serum is the medium of preference. Development of the latter on the serum-agar medium capped with sealing wax is very scanty.

The organism was successfully cultivated on plates. Bovine serum in a concentration of approximately 1:3 was added to nutrient agar just before solidification. When firm, several loops of a young culture were spread over the surface and the plate inverted. A film of growth was barely visible at the end of

twenty-four hours at 37°C. Distinct though very minute colonies were visible at forty-eight hours. Most of these showed little subsequent increase in size but a few attained a diameter of 0.5 mm. In the thicker portions of the plate the growth appeared to be continuous. Microscopical examination showed both discrete and coalesced colonies. The former were closely packed. The discrete colonies were circular, convex, finely granular, with smooth or slightly wavy margins. Capsules were not produced. Transfers from individual colonies to bovine serum agar showed the usual growth characteristics with typical capsule formation.

PATHOGENICITY OF THE ORGANISM

The pathogenicity of the rat form of *B. actinoides* was determined for the rat and for the guinea pig by several routes of injection. The reaction of the rat to the intra-aural administration of the organism is reported more completely elsewhere (Nelson, 1930). It was found that 75 per cent of a group of 30 young rats, two to three months old, showed a purulent exudate in the injected middle ear cavity. Sixty-five per cent of the rats gave pure cultures of *B. actinoides* from the affected tympanum. The organism was also recovered in cultures from the nasopharynx.

Intra-aural injection of the calf type of the organism was carried out with a smaller number of rats. Ten rats, two to three months old, were injected intra-aurally under ether anesthesia with small amounts of two strains.¹ The cultures were of recent isolation. The animals were held for a week and then killed. They appeared normal during this period. The injected middle ear cavities were normal in the gross in every case. Microscopical examination of saline washings showed large mononuclear cells, polynuclears, epithelial cells, and erythrocytes. The uninjected middle ear cavities were likewise normal in the gross. Washings showed epithelial and red blood cells with a very occasional polynuclear leucocyte. The presence of the macrophage type of cell, as shown elsewhere, (Nelson, 1930b) is a characteristic finding following the injection of sterile medium. The present

¹ Dr. T. Smith kindly supplied the calf strains of *B. actinoides* employed in this experiment.

series showed no quantitative difference in this type of cell following the introduction of the organism into the middle ear cavity. Most of the rats, however, showed more polynuclear leucocytes than are commonly found after the injection of medium. There is evidently a very slight cellular reaction to the presence of the calf type of *B. actinoides*. This is not visible grossly. *B. actinoides* was never recovered in cultures from the injected middle ear or from the nasopharynx on sealed coagulated bovine serum.

Intra-aural injection in the guinea pig was carried out only with the rat type of the organism. Six guinea pigs equally divided as to sex, and around 350 grams in weight, were anesthetized and injected in the left middle ear cavities with small amounts of a four-day-old serum agar culture of *B. actinoides*. Two rats, a male and a female, were killed at the end of two, seven, and fourteen days. No abnormalities were noted during these periods. The injected middle ear cavities of the two-day guinea pigs were normal grossly. Microscopical examination of saline washings showed numerous exudative cells, polynuclear and fewer large mononuclear cells. Epithelial and red blood cells were also present. The washings were definitely turbid in contrast to those from the normal uninjected cavities. *B. actinoides* together with an unidentified bacillus was cultivated from the injected tympanum of both guinea pigs. It was not recovered from the nasopharynx. Similar findings, save for the isolation of pure cultures of *B. actinoides*, resulted with the animals held for seven and fourteen days. The uninjected middle ear cavities were normal, grossly, and sterile in every instance.

Intraperitoneal and intrapleural injection of the rat type of *B. actinoides* had no effect on either rats or guinea pigs. Young rats only were employed for the former route, both young and adult animals for the latter. The guinea pigs were between 350 and 450 grams in weight. Single injections of 0.5 cc. and 1 cc. amounts of four-day-old cultures were made with both animal species. The animals were killed at intervals of one and two weeks after injection. The peritoneal and pleural cavities were

normal, grossly, in every instance. *B. actinoides* was not recovered in cultures from the spleen after injection by the former route or from the lung after the latter.

Subcutaneous injection of the organism produced local changes in both the rat and the guinea pig. With the former a small local abscess was formed which later retrogressed without breaking through the skin. Five young rats received 0.25 cc. each of a 4-day-old culture beneath the skin. They were killed at intervals of two, seven, ten, fourteen, and twenty-one days. Through the first ten days there was a small palpable module at the site of injection. The rat killed after two days showed a small circumscribed accumulation of purulent material. This was beneath the skin, embedded in the superficial tissue of the abdominal wall. At the end of seven and ten days, each of the rats showed a small, firm, walled off nodule under the skin at the site of injection. Purulent material was present on section. Each nodule was surrounded by a narrow red zone of congestion. They measured 8 by 5 mm. and 5 by 3 mm. respectively. In both cases *B. actinoides* was isolated from the sectioned nodule. The rats killed after two- and three-week intervals showed no palpable swelling at site. In each case beneath the skin was a small, brownish colored, slightly thickened area rather sharply demarcated. Microscopic examination showed fat globules, granular debris and a very occasional leucocyte. *B. actinoides* was not recovered in cultures from the first rat but was recovered from the second which was held for the longer period. The peritoneal and pleural cavities were normal throughout.

Subcutaneous injection in the guinea pig was likewise followed by local abscess formation but twice the volume of culture effective with the rat was required. Two guinea pigs were injected subcutaneously with 0.25 cc. of a four-day-old culture of *B. actinoides*. At the end of a week there was no palpable swelling at the site of injection and they were killed. The skin and abdominal wall were normal in both cases. Three additional guinea pigs were injected beneath the skin with 0.5 cc. amounts of a similar culture and killed at intervals of two, seven and ten days. In each case there was a palpable nodule at the site. The guinea

pig killed after two days showed at autopsy a small, firm, red-denied area beneath the skin. It was not purulent. *B. actinoides* and a staphylococcus were isolated in cultures. The animals, killed after seven and ten days, showed small, firm, walled off nodules under the skin at the site of injection. They were surrounded by a narrow red zone of congestion and on section yielded a small amount of creamy purulent material. A pure culture of *B. actinoides* was isolated in each case.

DISCUSSION AND SUMMARY

A close relation between the rat and calf types of the organism is indicated by the broadly similar nature of their growth characters outside the host, and in particular by their common production of a peculiar form of capsule. The basic morphological element of both appears to be a bacillus. A more detailed comparison of the two types, however, reveals a number of divergent characters which do not admit of their complete identity.

The rat type of the organism is somewhat less specialized than the calf type in its growth requirements. The capping of a medium with sealing wax which tends to favor the surface growth of the latter has no effect on the development of the rat type. This organism grows equally well at the base and on the surface of sealed and unsealed culture tubes. A medium rich in serum constituents is essential for an unrestricted development of the calf type. Coagulated serum is the medium of preference for its cultivation. Nutrient agar enriched with fluid serum at the base fully meets the growth requirements of the rat organism.

Capsules are produced by both types under artificial conditions of growth but there is a difference in their number, shape, and distribution. The calf organism commonly forms club-shaped capsules tending towards a radial arrangement over the entire surface of the clump. Such capsules are less numerous with the rat organism and are often spherical in shape scattered at intervals throughout the clump. Cells with large central or terminal sporelike expansions which characterize the development of the rat type on the surface of serum-agar are not a morphological feature of the calf organism.

The direct introduction of the rat type into the middle ear cavity of the rat frequently gives rise to a purulent otitis with subsequent recovery of the organism in cultures. Intra-aural injection of the calf type in the rat calls forth a slight local cellular migration which is determined only by microscopical inspection. The organism is not recovered in cultures.

Because of the somewhat superficial nature of these divergent characters it seems unwise to establish a new species for the rat organism. It is possible that the two types are simply variants selected from a common strain by development in different hosts. If such were the case, however, a trend towards convergence would be anticipated with their continued cultivation under artificial conditions. Cultures under continuous cultivation for a period of eight months have shown no significant change in their growth characters. T. Smith (1921) has noted a decrease in virulence with a calf strain over a period of several years. For the present, the writer feels justified in regarding the rat organism as a fixed type and proposes to name it *B. actinoides*, variety *muris*.

This designation may be somewhat misleading in view of the earlier isolation by Jones (1922) of an organism resembling *B. actinoides* from the pneumonic lungs of albino rats. Cultures of this organism were not available for direct comparison. From the described characters, however, it bears a much closer resemblance to the calf type than to the present organism. The writer has also isolated *B. actinoides* from the lung in pneumonia but found it identical with the latter type. The exact position of the organism described by Jones with reference to the two types of *B. actinoides* under discussion cannot be defined.

It should be emphasized that the proposed name for the variety is not regarded as final. The biology of the organism is only imperfectly understood. It is possible that too much importance is attached to the various morphological elements. The usual immunological methods for differentiation are not readily applicable to a microorganism of its nature. Up to the present, attempts to produce an antiserum reactive with the present type have met with failure. The isolation and comparative study of

B. actinoides from other animal sources would materially assist in establishing relationships within the species.

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INFLUENCE OF OSMOTIC PRESSURE UPON SPORE GERMINATION

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Received for publication, September 24, 1930

The work of Ruehle (1923) and Magoon (1926) shows clearly that bacterial spores are not entirely dormant but possess rather a sluggish enzymic activity. In the spore form, as Magoon suggests, the vital cell processes are retarded but not completely suspended. Notwithstanding this fact, nongerminating spores are relatively innocuous and quite incapable of producing significant alterations in their environment. Extensive changes through the agency of spores can occur only by the process of germination and subsequent vegetative development. In food materials, therefore, spores assume practical significance only when environmental conditions permit their development. Effective control of germination is of particular importance in those canning operations which do not effect complete sterilization, for in these cases continued preservation rests largely upon the maintenance of conditions unfavorable to the germination of the surviving spores.

Present efforts to control germination are based almost wholly upon our general knowledge of the principles of biologic behaviour. The particular factors which specifically regulate or modify the germinative process are not clearly understood and the influence and relative importance therein of many physical and chemical forces has received but little attention in the literature. As basic information of this nature must be acquired before successful control measures can be established, an effort has been made in this paper to contribute to our limited knowledge upon this subject.

Bacteria, in common with all plant and animal cells, are pro-

foundly influenced by the osmotic pressure of their fluid environment. The enveloping wall or pellicle which is believed to surround both vegetative and spore cells appears to function as a semi-permeable membrane, freely permeable to water but manifesting a considerable degree of specificity toward other substances including salts and organic constituents. While absorption through membranes of this nature is no longer considered to be solely regulated by differences in osmotic pressure, the importance of this factor in modifying the absorption process can hardly be questioned. Spores, because of their condensed constitution, are probably somewhat less reactive to osmotic changes than vegetative cells. The concentration of protoplasm incident to sporulation increases its viscosity and this retards the molecular movements, which bring about an osmotic equilibrium. Aside from a slower response, however, there is probably no fundamental difference in the reaction of spores and vegetative cells to osmotic forces. The few studies which have correlated spore development with osmotic changes suggest that this factor may play an important rôle in the process of germination.

METHODS

Organism. Following preliminary experimentation with several aerobic spore bearers, *B. mycoides* was chosen as being especially suitable for spore studies. The particular strain used was obtained from Dr. C. A. Magoon, Bureau of Plant Industry.

Culture media. Varying concentrations of plain infusion broth, extract broth and peptone solutions constituted the nutrient media. The solutions were adjusted to pH 7.0 if necessary, and were made up in sufficient quantity so that the same lot was used throughout the study of each particular factor.

Preparation of spore suspension. The spore suspensions were prepared by washing the surfaces of agar slopes with sterile distilled water and filtering through sterile absorbent cotton to remove particles of agar. The strained suspension was then centrifuged at high speed to throw down the cells, and the spores were resuspended in sterile distilled water. This process was repeated to remove the last traces of residual nutrients and metabolic

products. After decanting the final wash water the spores were diluted with distilled water to a desired opacity using an aqueous suspension of barium carbonate as the standard.

Germination technique. Osmotic effects were studied by the hanging drop culture method in which straight sided cells were employed as incubation chambers. A drop of the test fluid placed at the base of each cell served to reduce evaporation and hastened the establishment of an equilibrium in vapor pressure between the drop and the enclosed atmosphere. Uniform sized drops of the test and control solutions were placed upon separate, clean, sterile coverslips and then rapidly inoculated with a straight needle dipped into a spore suspension. The spores were thoroughly mixed with the medium and spread evenly over a small area. The coverslip was then inverted over the cell and sealed by means of melted paraffin. The cultures were examined immediately for the proper number and distribution of the spores and then placed in an incubator providing a temperature of 30°C. Microscopic observations were made at regular intervals, using the oil immersion objective.

The rate of germination was determined by counting one hundred spores and noting the percentage of these which were in the process of germination. Experience indicated that this procedure yielded representative results with a minimum time factor. While the process of germination is visibly initiated by a swelling of the cells and loss of refractivity the rupture of the spore envelope was chosen arbitrarily as the first criterion of germination. Cells in the outer zone of the microscopic field were not counted because of the imperfect focus obtained. The first evidence of rupture of the spore envelope was detected without difficulty by slightly varying the focus of the field by means of the fine adjustment screw. The use of a projection arc lamp using a 500 watt bulb greatly facilitated this procedure.

It is possible, of course, that the intensity of the arc light may have exerted a slight influence upon the germination process, but inasmuch as the exposures were brief and of equal duration this effect may be considered negligible.

The reduction in oxygen supply incident to the sealing process

in the germination technique led to a study of the possible effect which this condition might have on the course of germination. As a control, an unsealed inoculated preparation was arranged so that the cover slip only partially closed the depression in the hanging drop cell, thus insuring an abundant oxygen supply. The test preparation was sealed as usual and both preparations incubated at the same temperatures. Under these conditions repeated trials extending over the usual periods of observation gave identical results, which indicates that the oxygen supply in the closed cells was ample for optimum germination.

Osmotic measurements. The osmotic pressure values were obtained indirectly by determining the depression of the freezing point, and from this calculating the osmotic pressure in atmospheres.

RESULTS

The germination of spores was first studied through the range of osmotic concentration at which most biologic forms thrive best. Five cubic centimeter portions of plain infusion broth were diluted with sterile distilled water and the osmotic pressure regulated by the addition of small amounts of sterile 10 per cent sodium chloride solution. As indicated in the protocol, the quantity of broth and volume of fluid remained constant in all cases.

The comparative rate of germination in four of these solutions is shown in table 1. Because of the difficulty of making accurate observations on four solutions simultaneously, the results represent the general average of many experiments in which two solutions were compared at one time. While marked variations could not reasonably be expected, a very large series of these studies revealed consistent differences in the rate of germination as shown in this table. Germination was always most rapid at the lowest osmotic pressure. As the osmotic tension of the solution was progressively elevated, the rate of germination became correspondingly slower. The total percentage germination was unaffected under these conditions. The observed effects can hardly be attributed to a specific salt action in view of the small amount of the substance used. The highest concentration of salt

was only slightly greater than that present in physiological salt solution. While it was not expedient in this experiment to lower the osmotic pressure further, there is no reason to suppose that the lowest pressure represents the lowest compatible with optimum germination. The results in general show that optimum germination occurs in solutions having a relatively low osmotic pressure.

In order to study germination at lower osmotic concentrations, a 1 per cent solution of peptone was progressively diluted with sterile distilled water until a series of six concentrations was ob-

TABLE 1
Germination of spores in diluted infusion broth at different osmotic pressures

TIME FROM INOCULATION	GERMINATION			
	cc. distilled water = 1.9	cc. distilled water = 1.6	cc. distilled water = 1.5	cc. distilled water = 1.3
	cc. 10 per cent NaCl = 0.1	cc. 10 per cent NaCl = 0.4	cc. 10 per cent NaCl = 0.5	cc. 10 per cent NaCl = 0.7
	Osmotic pressure atmospheres = 9.17	Osmotic pressure atmospheres = 12.92	Osmotic pressure atmospheres = 13.52	Osmotic pressure atmospheres = 15.81
<i>minutes</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
45	6	3	1	1
55	21	18	12	5
60	72	58	48	32
70	84	82	74	75
80	90	90	85	85
90	91	90	90	90

tained. Each of these was then divided into two equal portions; one from each dilution then received sufficient sodium chloride to lower the freezing point about 0.4° . The samples were resterilized and freezing point determinations made. This procedure yielded a series of increasingly dilute peptone solutions, in each concentration of which there were two solutions differing only in their osmotic pressure. By slightly varying the amount of salt added to the adjusted solutions, the osmotic pressure variations were made nearly identical throughout the series. The germination of the standard spore suspension was tested under these conditions and the results assembled in table 2a and 2b.

In the highest dilution of peptone, germination failed to occur in either solution during the arbitrary incubation period of three days. However, this concentration was just barely below the

TABLE 2A

Comparative germination of spores at different osmotic pressures in very dilute peptone solutions

TIME FROM INOCULA- TION	GERMINATION		TIME FROM INOCULA- TION	GERMINATION		TIME FROM INOCU- LATION	GERMINATION	
	Percentage peptone = 0.16			Percentage peptone = 0.09			Percentage peptone = 0.05	
	NaCl per cent = 0	NaCl per cent = 0.60		NaCl per cent = 0	NaCl per cent = 0.60		NaCl per cent = 0	NaCl per cent = 0.60
	Osmotic pressure atmos- pheres = 1.20	Osmotic pressure atmos- pheres = 8.21		Osmotic pressure atmos- pheres = 1.44	Osmotic pressure atmos- pheres = 6.40		Osmotic pressure atmos- pheres = 1.20	Osmotic pressure atmos- pheres = 6.40
minutes	per cent	per cent	minutes	per cent	per cent	minutes	per cent	per cent
55	4	0	70	8	0	75	0	0
65	35	20	105	21	0	120	5	0
85	87	76	205	58	0	160	7	0
105	93	90	235	60	0	210	22	0
130	95	95	3 days	61	14	3 days	50	7

TABLE 2B

Comparative germination of spores at different osmotic pressure in very dilute peptone solutions

TIME FROM INOCULA- TION	GERMINATION		TIME FROM INOCULA- TION	GERMINATION		TIME FROM INOCU- LATION	GERMINATION	
	Percentage peptone = 0.03			Percentage peptone = 0.025			Percentage peptone = 0.020	
	NaCl per cent = 0	NaCl per cent = 0.66		NaCl per cent = 0	NaCl per cent = 0.66		NaCl per cent = 0	NaCl per cent = 0.73
	Osmotic pressure atmos- pheres = 1.20	Osmotic pressure atmos- pheres = 6.76		Osmotic pressure atmos- pheres = 1.20	Osmotic pressure atmos- pheres = 6.87		Osmotic pressure atmos- pheres = 1.08	Osmotic pressure atmos- pheres = 7.24
	days	per cent		per cent	days		per cent	per cent
3	35-40	0	3	20	0	3	0	0

nutrient minimum for germination, as indicated by the fact that an increase in peptone of 0.005 per cent accompanied by an almost imperceptible change in osmotic pressure, permitted some germi-

nation. Curiously enough the untreated solution with the lower osmotic pressure produced some germination, while the salt regulated solution prevented germination. In the next higher concentration the same general type of result was obtained. The germination was somewhat greater in the plain peptone solutions and, again, entirely prevented in the solution containing a small amount of salt. Germination occurred in both solutions in the next lower dilution though the added sodium chloride again exerted a marked inhibitory action. With increased concentration of peptone the same salt concentration becomes less and less inhibitory, until in the lowest dilution only the rate of germination was affected. In order to determine whether the observed effect was due specifically to the sodium ion, potassium chloride was substituted in one concentration. Results of a similar nature were obtained in this case though the potassium salt seemed to be somewhat less toxic.

Under these conditions it appears that 0.025 per cent peptone represents the minimum quantity that will support germination. The lower minimum concentration of a mixture of peptone and beef extract reported by Pringsheim and Langer (1924) is in harmony with results obtained later, in which infusion broth was found to be much more favorable to germination than peptone solutions alone. These observations emphasize the value of meat extractives in promoting germination. In these extremely dilute solutions lack of sufficient food is probably the limiting factor rather than unfavorable osmotic conditions, as indicated by the fact that elevation of the osmotic tension by means of neutral salts in no case increases either the rate or percentage of germination. The enhanced toxicity of the salts evident in these high dilutions, however, quite obviously complicates an interpretation of these results and may mask completely the purely osmotic effects. The inhibiting action associated with such small quantities of sodium chloride in these dilute solutions suggests that peptone functions as a buffer in reducing the chemical toxicity of the salts. Thus, with increasing dilution of peptone a given salt concentration becomes progressively more toxic. This is shown by a comparison of the ratios of germination between the plain peptone

medium and the salt peptone medium in table 2a. In the highest concentration of peptone, the ratio of germination in the two solutions was 1:1, the difference being only in the rate of germination. In the next two lower peptone concentrations, however, the ratios of germination in corresponding solutions were approximately 1:4 and 1:7 respectively. Sodium chloride was apparently somewhat more inhibitory than isotonic potassium chloride though the normal salt content of the peptone may have influenced this result. The protective action exerted by the peptone may be correlated with the presence of antagonistic salt elements in the medium, as Loeb (1919), Lille (1919) and other investi-

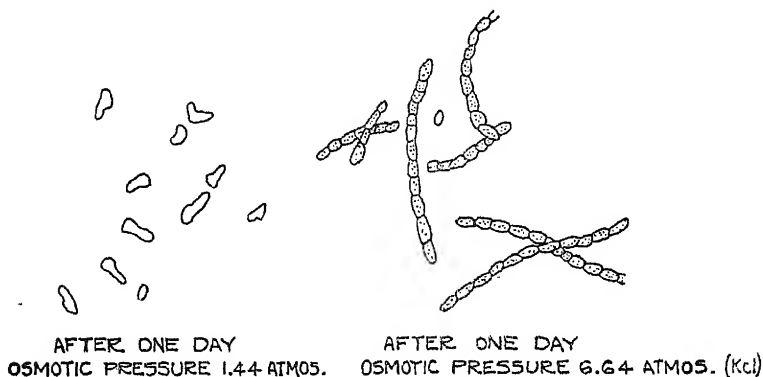


FIG. 1. GERMINATION OF *B. mycoides* IN 0.09 PER CENT PEPTONE

gators have shown that polyvalent cations diminish the toxicity of sodium chloride solutions toward bacteria and other microscopic organisms. The germination which occurred in the unregulated solutions proceeded only to the formation of short rods. In contrast to this, the corresponding solutions with the osmotic pressure elevated, revealed long rods and filaments. This was true in the presence of both salts and also when an unfermentable sugar, was employed as the osmotic agent. This latter observation is rather conclusive evidence that the optimum osmotic pressure for vegetative growth is definitely higher than that required for germination. Camera lucida drawings of the germination in the potassium chloride solution are shown in figure 1.

The exact osmotic pressure which will provide optimum conditions for germination varies with the nature of the nutrient medium as indicated by the following experiment. Two solutions of infusion broth were diluted with sterile distilled water until the depression of their freezing points corresponded exactly with that of 1 per cent and 0.5 per cent peptone solutions respectively. Germination in these solutions is shown in table 3. It is evident from these results that infusion broth sustains maximum germination at a lower osmotic pressure than peptone alone. Lowering the concentration of peptone from 1 per cent to 0.5 per cent appar-

TABLE 3

Germination in infusion broth and peptone water at the same osmotic pressure

TIME FROM INOCULATION	GERMINATION			
	1 per cent peptone	0.5 per cent peptone	Infusion broth	Infusion broth
	Osmotic pressure atmospheres = 7.36	Osmotic pressure atmospheres = 6.52	Osmotic pressure atmospheres = 7.24	Osmotic pressure atmospheres = 6.52
<i>minutes</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
45	13	1	20	18
60	80	8	85	81
80	85	38	91	89
95	90	47		
170		73		
380		88		

ently reduces the concentration of available nutrients below the minimum necessary for optimum germination. In the infusion broth, it is presumed, the nutrients are more suitable or readily available and consequently a smaller amount will suffice to meet the minimum food requirement for germination.

The development of spores in solutions of higher osmotic tension was now considered. The germination in one and ten per cent peptone solutions is shown in table 4. When the quantity of food was increased ten fold the rate of germination was considerably retarded. This effect may be ascribed to the unfavorable osmotic pressure created by the higher concentration of peptone. It appears that when the food supply is definitely in excess of the

minimum required for optimum germination, a certain degree of dilution will accelerate the rate of germination due to the production of a more favorable osmotic pressure. If, however, the con-

TABLE 4
Germination of spores in 1 and 10 per cent peptone solutions

TIME FROM INOCULATION	GERMINATION	
	1 per cent peptone	10 per cent peptone
	Osmotic pressure atmospheres = 7.24	Osmotic pressure atmospheres = 14.37
minutes	per cent	per cent
45	7	3
60	34	9
75	87	27
90	89	63
110	90	82
130	91	86
190	91	90

TABLE 5
Limiting peptone and neutral salt concentrations for the germination of spores

SUBSTANCE	PERCENTAGE	pH	OSMOTIC PRESSURE ATMOSPHERES
NaCl.....	5.5	6.93	46.24
KCl.....	6.5	6.94	46.12
NH ₄ Cl.....	4.0	6.73	38.76
CaCl ₂	1.8	6.90	12.80
MgCl ₂	1.8	6.79	12.55
NaBr.....	6.5	7.13	36.00
NaNO ₃	7.0	6.88	39.95
Na ₂ SO ₄	5.0	7.02	20.76
Na ₂ HPO ₄	4.7	7.06	17.26
Na ₂ C ₂ H ₃ O ₂	9.0	7.26	40.09
Na ₂ C ₆ H ₅ O ₇	3.5	7.40	10.86
Peptone.....	35.0	7.00	39.60

centration of nutrients is barely sufficient to support optimum germination as was the case in the 1.0 per cent peptone solution, dilution of this medium and consequent lowering of the osmotic pressure results in a retarded rate of germination despite the fact

that the osmotic pressure induced by the dilution may support optimum germination under different nutritional conditions.

Holzmüller (1909) also studied spore germination in different concentrations of infusion broth and noted some of the relationships just described. There was also evidence of a minimum osmotic pressure for optimum germination, which was not observed in our experiments. The author's conclusion that osmotic effects exert greater influence upon germination than the nature of the nutrient material must be accepted with reservation, due to the fact that the variable factors in this particular experiment were not limited to the one under consideration.

The results just describe led to a study of the limiting osmotic pressure for germination. A series of neutral salts when incorporated in 1 per cent peptone solutions prevented germination at the pressures indicated in table 5. The limiting peptone concentration is also included.

The reaction was adjusted only in the case of the ammonium chloride and phosphate solutions as the final pH of the others was very near the neutral point. Examination of these figures indicates that there is a rather definite limiting osmotic pressure for germination. This corresponds roughly to 36–46 atmospheres. When other factors do not intervene germination ceases within this range. In some cases inhibition of germination was obviously brought about by the chemical activity of the salt. The bivalent salts are especially powerful in this respect. In these cases the solution becomes toxic before the limiting osmotic pressure is reached. The comparatively narrow limiting osmotic range of most of the salts and peptone shows that adverse osmotic conditions prevented the germination in these cases. This result concurs with that obtained by Eijkman (1918) and demonstrates the existence of a limiting osmotic pressure for germination in concentrated solutions.

In one of the preceding experiments, the nature of the nutrient material in dilute solution was shown to determine the osmotic tension at which optimum germination takes place. In order to determine whether this factor influences the limiting osmotic pressure the inhibiting concentration of sodium chloride infusion

broth was compared with that of the sodium chloride peptone solutions. The former limiting tension was found to be 46.85 atmospheres. This corresponds closely to the limiting sodium chloride peptone concentration. Germination was thus prevented at the same osmotic pressure in the two solutions, despite the fact that the percentage of sodium chloride in the two solutions was dissimilar. This observation furnishes additional evidence of the existence of an inhibiting osmotic tension and indicates that the nature of the nutrient material does not influence this limiting pressure.

While it appears reasonable to suppose that these results may apply to bacterial spores in general, the following summary refers specifically to the spores of *B. mycoides* under the prescribed conditions of these experiments.

SUMMARY

1. Germination of spores is most rapid and complete at relatively low osmotic pressure.

2. The osmotic tension necessary for optimum germination is definitely lower than that required for maximum vegetative development.

3. The exact osmotic pressure at which optimum germination occurs depends upon the nature of the nutrient material, the more suitable or readily available the food the lower the osmotic pressure at which optimum germination occurs.

4. There is a definite minimum concentration of food below which no germination occurs, irrespective of the osmotic pressure. In the case of peptone solutions this was found to be 0.025 per cent.

5. A deficiency in food is probably the limiting factor in most low pressure solutions.

6. In these experiments there was no evidence to indicate a purely osmotic limitation in low pressure solutions.

7. When the minimum nutritional requirements for optimum germination are exceeded, an increased concentration of food may retard the rate of germination due to the unfavorable osmotic pressure created by the added nutrients. Under such conditions a certain degree of dilution will accelerate the rate of germination.

Conversely, increasing the food supply increases the rate of germination only when the nutrient concentration is below the critical minimum.

8. There is a rather definite limiting osmotic pressure for germination. This corresponds roughly to 36–46 atmospheres. In the absence of other limiting factors, germination is inhibited within this range.

9. Certain substances which are non-toxic to spores in usual nutrient concentrations are extremely inhibitory in dilute solutions.

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INFLUENCE OF SURFACE TENSION UPON THE GERMINATION OF BACTERIAL SPORES

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Received for publication September 24, 1930

In the bacteriological literature of recent years, much attention has been devoted to the study of surface tension in relation to vegetative development. Despite widespread interest in this subject the relative importance of surface forces in bacterial growth remains unsettled. Misinterpretation of results appears to have led to exaggeration of the significance of this factor in many cases. Nevertheless, it seems clearly established that the vegetative development of some types of bacteria is profoundly influenced by variations in the surface energy relationships of their environment. Evidence of this nature, together with the fact that previous researches upon surface tension were confined exclusively to vegetative cells, seemed to be sufficient justification for including this factor in a study of spore germination.

If spore germination were dependent upon diffusion of nutrient or stimulating substances through the cell membrane, we might reasonably expect to find that surface tension would exert a definite influence upon germination, for, as Frobisher (1926) has pointed out, fluids of very low surface tension may under certain conditions be in more intimate contact with objects in suspension, and might therefore facilitate contact between the essential constituents and cell contents. Solutions of low surface tension in general penetrate or seep into microscopic spaces more rapidly than do fluids of high surface tension. Moreover, according to Billard and Dieulafe (1904), osmosis and diffusion are materially accelerated under certain conditions by lowering the surface tension of the solutions involved.

METHODS

Except where otherwise indicated the methods in this study were identical with those previously described (Curran, 1931). The same strain of *B. mycoides* was used.

Culture medium. Plain infusion agar adjusted to pH 7.0 constituted the basic medium.

Germination technique. The technique employed in studying surface tension involved the use of a solid substratum. Briefly, the procedure used was as follows: melted agar was poured into a petri dish having a minimum crowning at the center. Upon solidification of the medium and drying of moisture of condensation, small blocks approximately 5 mm. square were cut in the test and control agar with a small sterile knife. A small loopful of the spore suspension was then spread evenly upon the surfaces of the two blocks. Allowing one to three minutes for the culture film to dry, the inoculated surfaces were placed face downward near the center of a clean sterile coverslip. The squares of agar were contiguous but not in direct contact. The coverslip was then suspended over a sterile straight-sided hanging drop cell, and the mount sealed by means of melted paraffin to prevent drying.

The rest of the procedure was similar to that already described.

Surface tension depressant. A 1 per cent solution of neutral sodium cleate (Merck) was used to reduce the surface tension of the culture medium. Sterilization was accomplished in the autoclave. The final reaction of the medium containing 0.1 and 0.5 cc. of this solution was approximately neutral. The higher concentrations produced a slight alkalinity.

Surface tension measurements. All the surface tension determinations were made with a du Nöuy tensiometer. Graded amounts of the sterile stock solution of oleate were added aseptically to 10 cc. quantities of the sterile agar and thoroughly mixed. The media, with and without depressant, were stored in the refrigerator until ready for use. This method of mixing depressant and media eliminated possible hydrolysis of the reagent during sterilization. By this method also, surface equilibrium was established before the measurements were made. The im-

portance of this factor in colloidal solutions has been emphasized by du Noüy (1924).

When the surface tension measurements were to be made, two tubes of agar—one oleate and the other plain agar—were dissolved in the Arnold sterilizer. The tubes containing the melted agar were then immersed in a hot water bath and the temperature reduced to 55°C. Using a sterile pipette, 1 cc. of the test solution was delivered upon the center of a flamed sterile watch glass. An interval of one-quarter to one-half of a minute was allowed to elapse, following which two or three determinations were made. Usually no difficulty was experienced in obtaining close checks with this procedure. The surface tension measurements were made between 50° and 52°C. When the measurements upon both solutions were completed, the agar was poured into separate petri plates and the procedure carried out according to the directions previously outlined.

Glassware. All glassware used in this experiment was cleaned by treatment with hot acid dichromate solution, and rinsed once with tap water and twice with distilled water,—the latter obtained by distillation in Pyrex containers in the presence of alkaline permanganate.

RESULTS

Owing to the negative nature of the findings, the data are presented in one condensed table (table 1). The figures represent the average of many experiments in which this type of result was consistently obtained. Reduction in the surface tension of 7 dynes from an initial measurement of 50 dynes exerted no significant effect upon the germination of the spores so cultured. The rate of germination and the total percentage of spores which germinated were essentially the same in both the test and control media. The slight discrepancy is entirely within the limits of error of the method employed. From this table it is seen that germination began after about forty-five minutes incubation under favorable conditions. Cell division, however, was not observed until the third hour. In the highest concentrations of oleate, vegetative multiplication was delayed an hour or more.

The fourth column presents the data secured when the surface

tension of the media was depressed to 35.1 dynes. In this case a very slight reduction in the rate of germination is manifest, but the total percentage of cells which germinated was practically the same in both cases.

A still further depression of the surface tension of the culture media yielded the results given in column 5. Reference to this table reveals a definite retardation in the germination rate and an appreciable reduction in the number of cells which germinated

TABLE 1
Showing the effect of surface tension upon the germination of the spores of
B. mycoides

TIME FROM IN- OCULATION	GERMINATION					
	No oleate added; 50.3 dynes per cm.	0.2 cc. 1 per cent oleate to 10 cc. agar; 43.4 dynes per cm.	0.5 cc. 1 per cent oleate to 10 cc. agar; 35.1 dynes per cm.	1.0 cc. 1 per cent oleate to 10 cc. agar; 32.8 dynes per cm.	2.0 cc. 1 per cent oleate to 10 cc. agar; 31 dynes per cm.	3 cc. 1 per cent oleate to 10 cc. agar; 30.7 dynes per cm.
minutes	per cent	per cent	per cent	per cent	per cent	per cent
30	0	0	0	0	0	0
45	8	8	5	4	2	2
60	44	43	30	14	5	4
75	82	79	63	38	11	9
90	93	89	72	67	24	21
120	94	91	80	79	45	32
150	95	93	84	81	55	33
180	96	94	85	82	59	34
240	96	95	91*	85*	61	36
300			94*	88*	65*	39
330					67*	42*

* Estimated.

during the period of observation. The retarded rate of germination is particularly pronounced during the initial hour and one-half of culture. Vegetative growth proceeded more slowly in the media of lowered surface tension, with the result that a longer time elapsed before the germinated spores attained the filament stage.

The depression in surface tension was carried to 31.0 dynes and 30.7 dynes in columns 8 and 7, respectively. The results in general are similar to those in the previous column, but indicate much greater retardation in rate of germination and reduction of viable cells.

The results of these experiments are plotted in figure 1. These curves bear a rather close resemblance to the vegetative growth curve. As shown in this figure, germination begins slowly, then passes into a short period during which the percentage rate of increase is logarithmic in nature. It then falls off rather sharply, following which further increases are gradual, extending over relatively long periods of time.

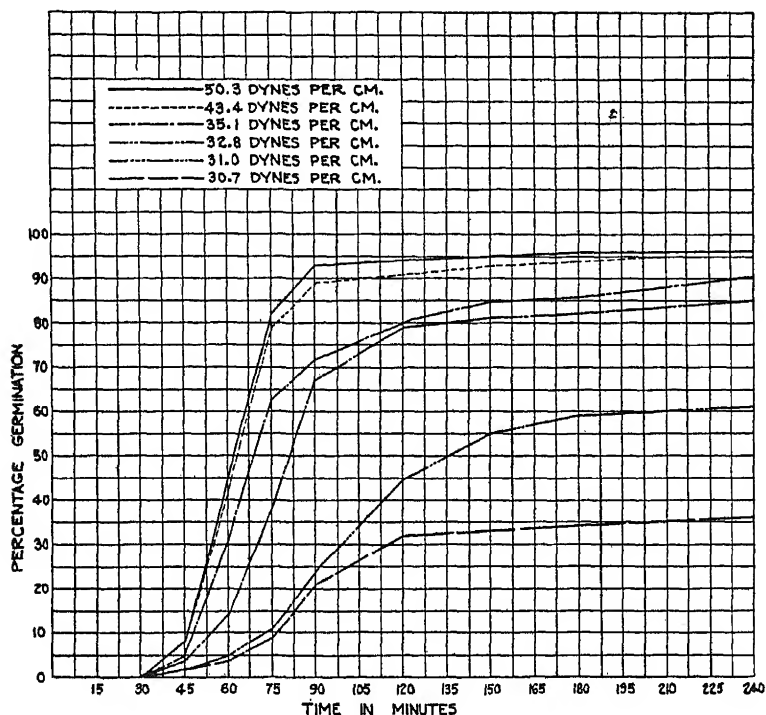


FIG. 1. GERMINATION OF THE SPORES OF *B. MYCOIDES* IN MEDIA OF DIFFERENT SURFACE TENSION

In the interpretation of these data the question may arise as to our justification in ascribing to surface tension observed effects when the possible chemical action of the depressant has not been excluded.

Sodium oleate employed as the surface tension depressant was

chosen as the compound which most nearly approaches the ideal substance. Except in relatively high concentrations, it is practically inert from the standpoint of nutrition and toxicity, and can be obtained in almost pure state.

Frobisher (1926) found a very slight growth of *A. aerogenes* in aqueous solution of sodium oleate, but it would seem illogical to attribute any significant nutritional effect on this basis, particularly in view of the fact that his triply distilled water supported some slight multiplication.

Avery (1918), Ayers, Rupp and Johnson (1923), Frobisher (1926), and Day and Gibbs (1928) utilized sodium oleate to study surface tension and their results indicate that with the exception of the particularly fastidious pneumococcus, and a very few strains of *Streptococcus pyogenes*, there was no appreciable restraining action in concentrations of 0.1 per cent and less. The work of Day and Gibbs (1928) reveals even a slight stimulative action in 0.1 per cent concentration upon the growth of *L. acidophilus* and *L. bulgaricus*. The observation of Ayers, Rupp and Johnson (1923) that sodium oleate is converted to an insoluble, inert form in the presence of acid would not invalidate the results herein reported because of the absence of any significant quantity of fermentable sugar in the germination medium.

Our own observations indicated that the vegetative cells of *B. mycoides* grew abundantly in nutrient solutions containing oleate in the highest concentrations used in the germination studies. Aside from these facts, however, the negative nature of the results obtained in the lowest concentrations of oleate, should constitute ample proof of the absence of significant chemical activity in these media.

DISCUSSION

The results described in the present paper indicate rather clearly that surface tension is not a significant factor in the germination of the spores of *B. mycoides* under normal circumstances. Organic acids, esters and other substances produced in the growth of many bacteria may, under certain conditions, reduce the surface tension of their solutions. Nevertheless the

depression in surface tension induced by purely metabolic activities would be considerably less than that produced by the lowest concentrations of oleate used in these studies. Within the conditions of the experiments the rate of germination was practically unaffected when the surface tension was reduced to 43.4 dynes, while a measurement of 35.1 dynes served only slightly to retard the rate of germination.

The total number of spores which germinated during the period of observation was not appreciably reduced until the surface tension was depressed to 32.8 dynes and, then, only slightly. With a longer period of observation it is not improbable that this slight difference would have entirely disappeared.

While germination proceeded quite as well with the surface tension reduced 7 dynes, as in the control, there is no evidence of stimulation. If the permeability of the spore envelope and diffusion of the nutrient fluid into the cell were increased, they exerted no appreciable stimulus to the germination process. It is conceivable, of course, that other interacting factors may have masked possible effects upon permeability and diffusion.

The rather marked decrease in the germination rate and reduction in viable cells evident in columns 4 and 5 was possibly due, in part at least, to the chemical activity of the depressant, for in these cases the oleate was present in concentrations of 0.2 and 0.3 per cent, respectively. However, if the lowered germination rate and total percentage germination were due primarily to the chemical factor, one should expect a far greater retardation in column 5, in which the concentration of oleate was more than 33 times greater than in column 4. While the difference is definite it appears to follow more closely the smaller change in surface tension than the concentration of the depressant. However this may be, the principal point which we believe this work demonstrates is that the spores of this organism are comparatively insensitive to relatively wide variations in surface tension. Reduction in the surface tension representing 15 dynes produced but slight influence upon the germination process, while, with the maximum possible depression, 50 per cent of the spores germinated within six hours.

In the interpretation of these data it must be recognized that the results reported, in common with those of other investigators, are based upon measurements of the surface tension at the air-medium interface which may or may not represent the surface energy relationship at the organism-medium interface.

The facts brought out in this study may be briefly summarized as follows:

1. A reduction in surface tension to 43.4 dynes from an initial of 50 dynes has no appreciable effect upon either the rate of germination or the total percentage of viable spores.

2. The development of spores in media the surface tension of which is depressed to 35.1 dynes is only slightly affected. The rate of germination is slightly retarded and the total percentage of germinating cells is somewhat less over a five-hour period.

3. At 32.8 dynes the rate of germination is markedly retarded but the total percentage of viable spores is only slightly reduced.

4. Below 32.8 dynes, germination is attended by a material decrease in both rate and total germination.

5. With the surface tension reduced to 30.7 dynes about 50 per cent of the spores are able to germinate within six hours.

6. Depression of the surface tension can not be relied upon to prevent or accelerate spore germination under ordinary conditions.

7. Surface tension is not a significant factor in the germination of the spores of *B. mycoides*.

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AN IMPROVED METHOD FOR THE DETECTION OF HEMOLYTIC STREPTOCOCCUS CARRIERS¹

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Received for publication October 8, 1930

In preparation for a survey of carriers of hemolytic streptococci among the nursing and medical staff of an obstetrical hospital in relation to occasional cases of sepsis, two difficulties of a purely technical nature were encountered, and a simple method was developed for avoiding the errors to which they led. While we claim no great originality for the procedure as finally adopted, it has proved to be so uniformly satisfactory in repeated culturings of large groups that it seems worth while to present it briefly with a discussion of the reasons for its use. A consideration of the difficulties may well be grouped under two heads: (1) the culture media used, and (2) the confusion caused by the presence of the *Hemophilus hemolyticus* in many throats.

MEDIA

In this laboratory, during the course of several years, the preparation of blood agar plates for teaching and research purposes and for occasional diagnostic use has become more or less standardized. A basic agar, made with slight modifications like the "hormone" agar of Huntoon (1918), is always used. To this, is added horse blood, since it happens to be easily available in large quantities. This medium is as readily prepared as any agar-containing meat infusion, and would seem to be optimal for most of the pathogens ordinarily cultivated on blood agar. However we have had trouble with it at intervals, particularly with the streptococcus group, for a long time and were inclined to attrib-

¹ The expenses of this study were defrayed by a grant from the DeLamar Mobile Research Fund.

ute the difficulty to varying specimens of horse blood employed. The blood is obtained through the courtesy of the Director of Massachusetts Antitoxin Laboratory, often probably from horses which have been partially immunized to pneumococci or streptococci, and frequently the proportion of cells to plasma is low. This difficulty was exceedingly troublesome at the time that the contemplated survey of normal throats was begun. The question of blood was first investigated. We took pains to get fresh blood from normal horses and also used, on a smaller scale human, sheep and rabbit blood by way of comparison. However, it appeared that the blood was not entirely, or even largely to blame, but that the difficulty lay with the basic hormone agar. It seemed impossible for our media kitchen to supply two consecutive lots of agar on which a series of hemolytic streptococci would behave similarly. Every gradation of hemolysis, from none at all to the typical wide zone, would be produced by the same strain on successive lots of media. Whether this was due to uncontrollable variations in muscle sugar or to some other difference, was not determined, but it was obviously necessary to simplify the agar base to the point where successive lots could be made uniformly.

In a study made some years ago by one of the writers (Mueller, 1922) of the cultural requirements of hemolytic streptococci, it had been shown that the quantity of meat habitually used in the preparation of infusion media, for this organism at least, was very greatly in excess of the amount necessary to give optimal growth. Small lots of agar were therefore prepared in which the meat was cut down to one-fifth or less of the usual amount, i.e., 100 grams or less per liter, instead of 500 grams. On such media, the uniformity of growth with successive batches proved to be entirely satisfactory, and, finally, an agar containing no meat infusion whatever was used. Parke-Davis peptone 1 per cent, salt 0.8 per cent and agar 1.5 to 1.8 per cent dissolved in water, titrated to pH 7.6, to which was added 10 per cent horse blood, supported excellent growth of all hemolytic strains tested, and the zone of hemolysis was sharp and wide. In the last eight months we have used many lots of this media, invariably with entirely satisfactory results. Less experience has been had with its use for other

organisms, but apparently it is quite suitable for *Streptococcus viridans* and pneumococci. One strain of gamma streptococcus grown on it has produced colonies indistinguishable from the alpha type, but with this exception other organisms studied have been typical.

HEMOPHILUS HEMOLYTICUS OR BACILLUS X

The presence of a Gram-negative bacillus producing hemolysis on blood plates was described by Pritchett and Stillman (1919) in a study of the occurrence of *H. influenzae* in throats following the epidemic of 1918-1919. Their difficulty lay in the separation of this organism from the influenza bacillus on oleate or chocolate agar, and they do not stress the question of differentiation from hemolytic streptococci, although their plate (fig. 5, plate II) shows quite well the similarity in appearance on blood plates. Practically, on a blood plate inoculated from a throat swab, crowded with colonies, it is impossible to tell the difference between the colonies of this gram negative organism and hemolytic streptococci. With pure cultures, lightly streaked out, differences are more apparent, but we venture to state that gross inspection of blood plates for hemolytic streptococci from throat cultures must inevitably lead to erroneous findings if the *Hemophilus hemolyticus* is present, and that, where no means are taken to insure its absence, the findings are valueless. It appears to occur sporadically in a large proportion of throats in a given group of people, and after a few months may almost completely disappear, and, still later, be found again. During November and December of last year, nearly 60 per cent of the normal throats we cultured showed from a few to several hundred of these colonies on a blood plate. In the spring of this year, none were found in the same group of individuals, and, recently, a few isolated plates have again shown them. Obviously, without some simple method of differentiation it is impossible to reach any sort of accurate knowledge of the occurrence of hemolytic streptococcus carriers in a large group.

Various attempts were made to assist in differentiation, and for the suggestion which led to the simple and entirely effective

method finally adopted we wish to express our thanks to Dr. S. B. Hooker of Boston University Medical School. It is based on the fact that hemolytic streptococci remain viable for considerable periods of time in broth of sufficient alkalinity to destroy many other bacteria. Not only is the *Hemophilus hemolyticus* readily killed in this way, but apparently many other saprophytic throat organisms are also destroyed. The plates made by the method to be described frequently show nothing but *Streptococcus viridans* and *Str. hemolyticus*. We have not investigated to see whether pneumococci survive the treatment or not. We have never, in many hundred throat cultures, been troubled by the "Bacillus X" since adopting the technique, although control cultures have often shown its presence.

METHOD

A throat swab, inoculated in the ordinary way from the tonsils and back of the throat, is inserted immediately into a test tube containing 1.5 cc. of ordinary extract or infusion broth, and 0.3 cc. of a 1 per cent solution of anhydrous Na_2CO_3 . The carbonate is added by means of a sterile pipette after the broth has been autoclaved. The swab is allowed to soak in the alkaline broth for from one to two hours at room temperature, or may be incubated at 37° for half an hour. It is then stirred around in the tube, and a good sized drop transferred with the swab to the surface of one of the peptone-blood agar plates described above, and spread evenly over the surface with a capillary or wire spreader. After twenty-four hours' incubation, hemolytic colonies are clearly visible, and may be fished and streaked out for isolation on fresh blood agar plates.

DISCUSSION OF RESULTS

Hemolytic colonies isolated in this way have invariably proved to be streptococci. Experiment has shown that this organism will withstand considerably greater concentrations of Na_2CO_3 than that used, and for longer periods of time. There is consequently a large safety zone. Larger drops of inoculum may safely be used than when the throat swab is mixed with neutral broth, and

in parallel tests, inoculating first from neutral broth and then adding alkali, allowing to stand and inoculating a second plate, it has much more frequently happened that two or three hemolytic colonies developed on the second plate and none on the first than *vice versa*. The number of hemolytic colonies obtained may vary from one or two up to practically a solid growth in different individuals.

Since repeated cultures have been obtained at bi-weekly intervals on a considerable group of healthy people, the validity of the method has been pretty well checked. A considerable number of individuals have been invariably positive on each of half a dozen consecutive cultures, and conversely many others are always negative. A more complete presentation of the results will form the subject of a later communication.

SUMMARY

A method for making *Streptococcus hemolyticus* carrier examinations has been described, in which a simplified blood agar and the selective removal of *Hemophilus hemolyticus* (Bacillus X of Pritchett and Stillman) by means of alkali have made possible results more accurate than we believe can be obtained by the usual procedure.

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THE EFFECT OF YEAST ON AMMONIA AND INDOL PRODUCTION BY BACTERIA IN CULTURE AND IN FECES SUSPENSION¹

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Received for publication, September 25, 1930

In a study of the effects of yeast ingestion on intestinal putrefaction, the results of which are to be published elsewhere, it had been noticed that when subjects on a constant diet were given yeast, there was an increase in the elimination of fecal ammonia and indol. The object of this bacteriological study was to obtain information which might serve as an explanation of these results.

The introduction of millions of yeast cells into the gastro-intestinal tract adds another complicating factor to the complex bacterial synergism already existent. Evidence that living yeast cells escape total destruction in the alimentary tract of man and animals has been presented by Neumeyer (1891), Haillon, Beylot (1896), Völtz (1919), and Rettger, Reddish and McAlpine (1924). Various aspects of the problem dealing with synergism of bacteria and yeasts have been illustrated and discussed in papers by d'Arsonval and Charrin (1893), Nobécourt (1900), Gerét (1901), Ledermann and Klopstock (1902-03), Volkova-Rubel (1916) and Castellani (1927). Vaughan, Novy and McClintock (1893) and Vaughan (1893) have shown that yeast nuclein exerts a germicidal action.

Ehrlich (1916) has shown that certain amines are deaminized almost quantitatively by yeasts, alcohol and ammonia being

¹ The material in this paper was submitted to the University of Rochester in partial fulfillment of the requirements for the degree of Doctor of Philosophy. This investigation was aided by a grant from the Fleischmann Company, now Standard Brands, Inc.

formed. Combe (1908) has suggested that yeast may weaken the toxins formed by intestinal bacteria, but he has given no positive proof. Dox (1917) and Effront (1908) have demonstrated the presence of deaminases in yeast, and the former investigator has pointed out that bacteria acting on amino acids produce either amines or organic acids.

The results of Gordon and McCleod (1926) and Kilborn, Pierce and Tittsler (1929) have shown that indol has a marked bactericidal action, this action varying with the microorganism under observation.

EXPERIMENTAL

Several bottles of Witte's peptone were mixed thoroughly to insure the use of the same peptone throughout the series of experiments. One and 2 per cent peptone water solutions, containing 0.5 per cent sodium chloride, were prepared. One hundred cubic centimeter portions of this peptone water were placed in 300 cc. Erlenmeyer flasks, and a sufficient quantity of extra water was added to each flask to take care of the average water loss during sterilization. Approximately 100 cc. of 1 or 2 per cent peptone water remained in the flask after sterilization.

To various lots of peptone water, prepared as mentioned above, amino acids, autolyzed yeast, and a commercial vitamin B preparation were added prior to sterilization. The amino acids were added to make 0.1 or 0.2 per cent solutions, and all dissolved with the exception of cystine. In all instances the determinations of amino nitrogen in the peptone water, to which pure amino acids had been added, yielded results averaging about 5 per cent less than the theoretical. This may have been due to moisture contained in the amino acid or to some alteration in the medium during the process of sterilization. The media containing amino acids were used in an effort to gain information as to the degree of deamination, but amino acids themselves affect bacterial growth, so that the results obtained were not entirely satisfactory.

The autolyzed yeast used was in the form of a dry, coarse powder which was ground until it would pass through a forty mesh sieve. Nothing had been added to this yeast to aid the autolytic

process. The powder did not go into solution, so that the peptone water appeared turbid.

The commercial vitamin B preparation was obtained from a local drug company, and had been received direct from the manufacturing laboratory shortly before its use. The powder was extremely hygroscopic and had to be weighed rapidly.

Solutions of several dyes were prepared in sterile water and when used were added to the peptone water solutions before inoculation with bacteria. These solutions were sterile.

Pure cultures of baker's yeast were grown on malt extract agar slants, and the yeast was suspended carefully by rubbing up the growth in sterile saline solution. These yeast suspensions were added to the peptone water before inoculation with bacteria. Certain of the suspensions were autoclaved before adding to the peptone water, so that the effect of living and dead cultures on bacterial growth could be determined. Between nine hundred million and a billion living or dead yeast cells were added to the peptone water when yeast was used. The various peptone waters were inoculated with suspensions of pure cultures of bacteria, or with feces suspensions prepared according to Torrey (1926).

The aeration method of Folin (1925) was used for the determination of ammonia, the aeration extending over a period of two hours. This time had been found sufficient for the removal of ammonia from the culture medium used. Ten cubic centimeters of the culture medium were used for all determinations, this volume being diluted to 75 cc. with sterile distilled water before the addition of potassium carbonate. Caprylic alcohol was used to prevent foaming. The amino nitrogen was determined by the formol titration method as given by Brown (1923).

Bergeim (1917) modified the Herter and Foster method (1905-06) for the determination of indol and Pierce and Kilborn (1929) have adapted Bergeim's method for use in determining indol in bacterial cultures.

Control experiments proved that there was little or no loss of ammonia or of indol during the period of incubation.

On account of the number of combinations of microorganisms and substances investigated, only a few tables containing representative results will be presented.

I. Effect of yeast on ammonia and indol production by pure cultures of bacteria (table 1)

Viable yeast, autolyzed yeast and the commercial vitamin B preparation increased ammonia and indol production by *B. coli-communis*. Cystine and glycine added to the medium had little or no effect upon ammonia formation, but these amino acids delayed indol production in the absence of yeast.

TABLE 1

*Ammonia and indol production in peptone water inoculated with B. coli-communis**

MEDIUM	AMMONIA PER 100 CC. MEDIA			INDOL PER 100 CC. MEDIA		
	72 hours	168 hours	384 hours	72 hours	168 hours	384 hours
	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
Peptone water.....	11	19	28	0	3.7	8.1
Peptone water + viable yeast.....	14	23	41	2.8	5.6	10.5
Peptone water + 0.2 per cent glycine...	11	19	37	0	3.3	10.4
Peptone water + 0.2 per cent glycine + viable yeast.....	12	25	53	2.5	4.9	10.5
Peptone water + 0.2 per cent cystine...	10	17	31	0	2.5	7.6
Peptone water + 0.2 per cent cystine + viable yeast.....	13	26	54	2.5	5.6	10.1
Peptone water + 0.2 per cent autolyzed yeast.....	10	18	35	0	4.6	9.9
Peptone water + 0.2 per cent commercial vitamin B preparation.....	11	25	39	2.9	6.6	11.8
Peptone water + 0.2 per cent commercial vitamin B preparation + viable yeast.....	19	33	62	0	3.4	9.5

* Studies with pure cultures of *B. proteus-vulgaris* yielded similar results or ammonia production, although the quantity of ammonia produced was greater

II. Effect of yeast on ammonia and indol production by feces suspensions (table 2)

Experiments planned to determine the range of variation of ammonia and indol production by suspensions of feces in the presence and absence of viable yeast showed that there was an average increase of 55 per cent in ammonia and 217 per cent in indol production attributable to the action of yeast. The condi-

tion and characteristics of organisms in suspensions of feces were too variable to permit the expectation of constant results.

III. Effect of yeast and amino acids on ammonia and indol production by feces suspensions (table 3)

Several series of experiments were carried out, and the results of one are presented in table 3. Yeast cells, living or dead, autolyzed yeast, and the commercial vitamin B preparation increased the rate of ammonia, amino nitrogen and indol formation early in the experiment. Later, there was no uniform effect.

TABLE 2

Variations in ammonia and indol formation in peptone water inoculated with feces suspensions in the presence and absence of yeast during a 72-hour period

NUMBER	AMMONIA PER 100 CC. MEDIA			INDOL PER 100 CC. MEDIA		
	Peptone water	Peptone water + yeast	Per cent increase due to yeast	Peptone water	Peptone water + yeast	Per cent increase due to yeast
	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
1	75	95	26.7	2.1	5.8	176
2	72	112	55.6	1.6	6.7	318
3	78	156	100.0	3.4	7.9	132
4	87	135	55.2	Trace	4.4	440?
5	100	134	34.0	6.0	8.2	37
6	85	135	58.8	2.3	6.9	200
Average..	83	128	55.0	2.6	6.7	217

Glycine, cystine, tyrosine and alanine retarded ammonia and indol production, probably through an inhibition of bacterial growth.

IV. Effect of dyes on ammonia and indol production by feces suspensions (table 4)

In an effort to determine what part of the fecal flora was responsible for increasing ammonia production, several dyes were used. Krumwiede and his coworkers (1918) showed that *B. coli-communis* did not grow in the presence of brilliant green. Churchman (1912, 1921-22) showed that acid fuchsin killed the more common Gram-negative, but not the Gram-positive bacteria,

TABLE 3

Effect of yeast on ammonia, amino nitrogen and indol in cultures with feces suspensions

MEDIUM	AMMONIA PER 100 CC.			AMINO N PER 100 CC.			INDOL PER 100 CC.		
	48 hours	120 hours	216 hours	48 hours	120 hours	216 hours	48 hours	120 hours	216 hours
	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
2 per cent peptone water.	26	138	181	10.15	72.96	31.43	2.99	8.70	12.42
2 per cent peptone water + viable yeast.	79	183	199	30.04	36.50	9.26	4.94	9.30	12.66
2 per cent peptone water + autoclaved yeast.	60	154	175	31.66	60.69	38.68	4.90	8.77	12.12
2 per cent peptone water + 0.2 per cent glycine..	14	113	157	-1.14	74.28	48.81	None	10.58	13.20
2 per cent peptone water + 0.2 per cent glycine + viable yeast.	65	191	213	20.88	20.41	7.61	4.33	11.17	13.80
2 per cent peptone water + 0.2 per cent glycine + autoclaved yeast.	39	171	194	19.34	48.55	23.93	3.45	10.98	14.34
2 per cent peptone water + 0.1 per cent cystine..	16	53	96	-10.76	8.14	18.71	None	5.73	10.87
2 per cent peptone water + 0.1 per cent cystine + viable yeast.	27	90	155	0.72	39.27	37.14	1.66	10.84	13.89
2 per cent peptone water + 0.2 per cent commercial vitamin B.	61	185	186	27.46	53.96	45.52	5.77	11.17	14.71
2 per cent peptone water + 0.2 per cent auto-lyzed yeast.	50	178	175	14.58	66.66	63.18	5.77	11.11	14.39
2 per cent peptone water + 0.2 per cent tyrosine.	13	119	114	No change	55.92	58.98	None	8.06	9.13
2 per cent peptone water + 0.2 per cent tyrosine + viable yeast.	62	156	139	27.30	55.68	46.97	5.66	8.59	9.35
2 per cent peptone water + 0.2 per cent alanine.	15	130	138	-2.86	62.76	54.92	None	8.77	10.15
2 per cent peptone water + 0.2 per cent alanine + viable yeast.	61	177	167	22.92	23.40	49.34	4.19	12.50	14.29

whereas gentian violet was found to prevent the growth of the more common Gram-positive spore-bearing aerobes, but not the Gram-negative organisms. The growth of *C. Welchii* was impeded by gentian violet, but the growth of *B. coli* was not mate-

TABLE 4
The effect of dyes on ammonia and indol formation by feces suspensions

MEDIUM	AMMONIA PER 100 CC.		AMINO N PER 100 CC.		INDOL PER 100 CC.	
	48 hours	96 hours	48 hours	96 hours	48 hours	96 hours
	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
2 per cent peptone water.....	8	35	-1.73	23.86	Trace	6.14
2 per cent peptone water + viable yeast.....	76	170	9.26	43.15	5.07	17.65
2 per cent peptone water + auto- claved yeast.....	24	130	8.04	65.51	2.97	9.76
2 per cent peptone water + gentian violet.....	6	9	0.20	4.37	None	Trace
2 per cent peptone water + gentian violet + viable yeast.....	12	127	3.29	30.23	Trace	8.13
2 per cent peptone water + gentian violet + autoclaved yeast.....	9	13	3.03	10.80	None	Trace
2 per cent peptone water + brilliant green.....	6	9	3.00	7.48	None	None
2 per cent peptone water + brilliant green + viable yeast.....	23	96	8.92	49.14	2.25	12.50
2 per cent peptone water + brilliant green + autoclaved yeast.....	8	64	4.57	28.07	Trace	10.46
2 per cent peptone water + 0.2 per cent commercial vitamin B.....	80	175	24.84	43.08	6.98	12.14
2 per cent peptone water + 0.2 per cent commercial vitamin B + gen- tian violet.....	24	121	9.33	70.59	2.41	9.22
2 per cent peptone water + 0.2 per cent commercial vitamin B + bril- liant green.....	9	14	12.33	15.11	None	None

rially affected. The concentration of the various dyes in the media was 1:300,000.

The ammonia and indol production was observed in several series of experiments, the results of one experiment being presented in table 4. A considerable reduction in bacterial activity

was produced by brilliant green and gentian violet, effectively preventing the formation of ammonia and indol. Acid fuchsin was inert at the concentrations used. Viable yeast always counteracted the bactericidal or bacteriostatic action of brilliant green and gentian violet. The dyes were decolorized by yeast, indicating decomposition of the dye molecule.

TABLE 5

Variations in the formation of ammonia, amino nitrogen and indol obtained on inoculating peptone water with feces suspensions in the presence and absence of yeast (aerobic)

MEDIUM	AMMONIA PER 100 CC. MEDIA		AMINO N PER 100 CC. MEDIA		INDOL PER 100 CC. MEDIA	
	48 hours	96 hours	48 hours	96 hours	48 hours	96 hours
	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
Peptone water.....	9	50	2	38	0.8	8.9
Peptone water + viable yeast.....	74	131	25	46	5.7	11.8
Peptone water.....	7	38	*	25	Trace	6.4
Peptone water + viable yeast.....	62	164	36	25	4.8	12.6
Peptone water.....	7	29	-2	24	Trace	6.1
Peptone water + viable yeast.....	62	140	9	43	5.1	17.7
Peptone water.....	6	18	1	19	Trace	3.9
Peptone water + viable yeast.....	68	110	28	68	5.5	13.4
Peptone water.....	49	119	22	58	6.1	8.9
Peptone water + viable yeast.....	86	134	34	38	6.9	10.8
Average in peptone water.....	16	51	5	33	1.4	6.8
Average in peptone water + yeast..	68	186	26	44	5.6	13.2

* No change.

V. Effect of anaerobiosis on ammonia and indol production

No definite effect was noted when the cultures were incubated under anaerobic conditions. The results of three series of experiments were contradictory, for in some cases anaerobiosis favored ammonia and indol production while in others production was greater under aerobic conditions.

VI. *Summary of effect of yeast on ammonia and indol production by suspensions of feces in peptone water (table 5)*

The results of comparable pairs of experiments, with values taken at the end of the forty-eight-hour and ninety-six-hour incubation periods, are summarized in table 5. The values showed a marked degree of variation, the greatest occurring in number 9 of the series where ammonia, and amino nitrogen, and indol were present in larger quantities than usual in a control. The effects of yeast are clearly shown by the average values at the foot of the table.

DISCUSSION OF RESULTS

Large variations were to be noted in the results obtained by the use of different feces suspensions. The suspensions were always prepared in the same way and the feces used were obtained following a day of uniform diet, the stool having been marked by use of charcoal. Variations in the daily excretion of viable bacteria can account for the lack of uniformity between the results obtained by use of different feces suspensions (MacNeal, Latzer and Kerr 1909a, 1909b).

The results reported in this paper show that living and dead yeast cells, autolyzed yeast, and a commercial vitamin B preparation, stimulate bacterial growth, viable yeast being most effective. Several explanations may be offered for the action of these agents.

The reaction of the media became more acid in the absence of yeast and the other preparations used and the increased acidity undoubtedly delayed bacterial growth for small quantities of ammonia and indol were found in the more acid media. It is probable that there is marked acid production early in the process of bacterial action, while little or no ammonia is available for neutralization. Yeast, by increasing the rate of deamination, would allow a plentiful supply of ammonia for acid neutralization. Possibly the buffering action of yeast protein itself plays a part in this process (Bokorny 1920).

Bacteria readily form amines during the decomposition of proteins or their derivatives; the extent of amine formation by bacterial cultures in the intestine is, however, not known. Yeast, in

causing a greater deamination may favor ammonia formation while limiting that of amines. Dox (1917) and Effront (1908) have demonstrated the presence of active deaminases in yeast. In our results, the greater formation of ammonia in media containing viable yeast suggests the presence of an active deaminase in yeast.

The proteolytic enzymes in the yeast cells may have caused a greater or more rapid digestion of the peptone, which in turn would permit an increase in the rate of deamination.

It seems evident that CO_2 formation should be increased in media containing yeast and this may account for the greater bacterial activity noted. Valley and Rettger (1927) and Valley (1928) have shown that bacterial growth is markedly affected by CO_2 . Fries (1906) called attention to the fact that the gas mixture in the intestine might serve as a regulator for the growth of microorganisms.

The stimulatory effect of the vitamin B complex is recognized. Robertson (1924), Johanson and Broadhurst (1926) and Werkman (1928) have discussed various aspects of the relationship between vitamin B and bacterial metabolism. Weight for weight, viable yeast was a more effective agent than the vitamin B preparation in promoting bacterial metabolism.

Gordon and McCleod (1926) found that glycine, cystine, phenyl alanine and tryptophane acted as inhibitory agents, while alanine favored bacterial growth and tyrosine was indifferent in its action. Our results show that cystine was most effective in delaying bacterial growth, while glycine, alanine and tyrosine also acted as inhibitory agents. Yeast tended to rule out this effect of the amino acids, possibly on account of the increased formation of ammonia, which reduced the acidity of the media.

Brilliant green and gentian violet retarded ammonia and indol formation. Brilliant green retarded indol production more than did gentian violet. The dyes were always decolorized in those media containing viable yeast, and the auxochrome groups of the dye may have been destroyed by yeast.

Indol, due to its bactericidal action, may have been responsible, at least partially, for some of the results observed. (Gordon and McCleod, 1926) (Kilborn, Pierce and Tittsler, 1929.)

The original data upon which the conclusions of this paper are based are on file in the Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, Rochester, N. Y.

CONCLUSIONS

1. The addition of living or dead yeast cells, particularly the former, to peptone water inoculated with pure cultures of *B. coli-communis* or feces suspensions resulted in an increased formation of ammonia and indol.

2. Yeast cells increased the rate of ammonia and indol formation, and deamination under the conditions of this experiment.

3. The results indicated that living yeast contains an active deaminase.

4. Brilliant green and gentian violet materially reduced bacterial growth, but yeast destroyed the toxicity of these dyes, at least partially. The commercial vitamin B preparation did not materially reduce the toxicity of these dyes.

5. The addition of a commercial vitamin B preparation to peptone water inoculated with feces suspensions resulted in a greater production of ammonia and indol but this preparation, weight for weight, was not as effective as living yeast.

6. The addition of certain amino acids to the media tended to delay bacterial growth. Cystine was the most effective, of the amino acids used, in retarding the growth of bacteria.

7. The results obtained under aerobic and anaerobic conditions were essentially the same.

8. Yeast appeared to increase bacterial metabolism. The amino nitrogen in several instances where living yeast was present in the media was low, but there was a correspondingly increased amount of ammonia, indicating that deamination occurred shortly after the amino nitrogen was available.

The author wishes to acknowledge his indebtedness to Professors Stanhope Bayne-Jones, John R. Murlin and H. A. Mattill for helpful suggestions and advice during the course of this investigation.

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STUDIES ON THE INFLUENCE OF BACTERIA ON THE OXIDATION-REDUCTION POTENTIAL OF MILK

I. INFLUENCE OF PURE CULTURES OF MILK ORGANISMS

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Received for publication October 11, 1930

The object of this work was to study the characteristic changes in the oxidation-reduction potential (Eh) of milk caused by pure cultures of milk bacteria. It was hoped that a knowledge of the relation of species and numbers of these milk organisms to the oxidation-reduction potential might be of value in the interpretation of results with milks in which these organisms predominate. This work must necessarily precede work, such as that reported in a following paper, on milk which contains more than one species of bacteria.

METHOD

For the potentiometric measurements, electrodes of stout gold foil were used. These were cut about 2 cm. square and welded to a platinum wire which was fused through the end of a glass tube. A few drops of mercury and a copper wire in the glass tube completed the electrode connection to the potentiometer. The electrodes and exposed platinum shafts were plated with gold from a gold cyanide solution. Whenever an electrode showed signs of improper functioning, it was cleaned of its surface gold by electrolysis and a fresh coating applied. The glass tubes of two electrodes were inserted in holes in a rubber stopper which fitted the 1-liter Erlenmeyer flasks to be used for the experiments. The electrodes were adjusted so that one would hang just above the bottom of the flask and the other about 3 cm. higher. By this adjustment it was possible to know in a general way whether

the measured potentials were being affected by diffusion of oxygen into the depths of the milk and to detect aberrant electrodes. Each stopper was also fitted with a short tube for sampling and venting and a syphon tube extending from the bottom of the flask to a somewhat higher level on the outside. The outer end of this tube was fitted with a short piece of rubber tubing. This tubing was closed with a pinch-cock, and both this and the vent tube were plugged with cotton previous to sterilization. The junction between milk and the saturated potassium chloride was made in a file scratch on the cock of a three-way stop-cock. The two non-connecting tubes of the stop-cock were connected one to the syphon from the flask of milk and the other to a syphon from the potassium chloride reservoir of the calomel half-cell. After milk and potassium chloride solution had alternately been drawn into the stop-cock, the cock was momentarily loosened and a satisfactory junction was formed. A Leeds and Northrop Type K potentiometer was used for all potential measurements.

The electrodes with the accessory syphon and vent were placed in a 1-liter Erlenmeyer flask half filled with water, sterilized at 120° C. and left in the tightly stoppered flask until needed. The glass stop-cocks were boiled in water just previous to use.

A 1-liter Erlenmeyer flask was filled about three-fourths full of fresh skim milk or of skim milk reconstituted from milk powder. In order to avoid possible variations in different samples of fresh milk, skim milk made as required from a single lot of skim milk powder was used in most experiments. Enough milk was placed in a smaller flask to make up the larger flask to its entire capacity. Both flasks were plugged with cotton and steamed for one-half hour on each of three successive days. Immediately after the third steaming the contents of the flasks were cooled to the temperature at which the experiment was to be conducted. The large flask was inoculated with 1 cc. of an eighteen-hour milk culture of the organism; the stopper containing the electrode assembly was inserted, and the flask was filled to the neck with milk from the smaller flask.

The larger flask was then immersed to its neck in water in a 3-liter beaker and the water was kept within one degree of the

temperature desired. The syphon was connected via the three-way stop-cock to a syphon from the saturated potassium chloride reservoir of the calomel electrode and the electrode wires were connected to the potentiometer switches. Readings of oxidation-reduction potential and hydrogen-ion potential were made at suitable intervals.

Direct microscopic counts were made on the milk by the Breed method. The counts were usually made on dilutions of the milk

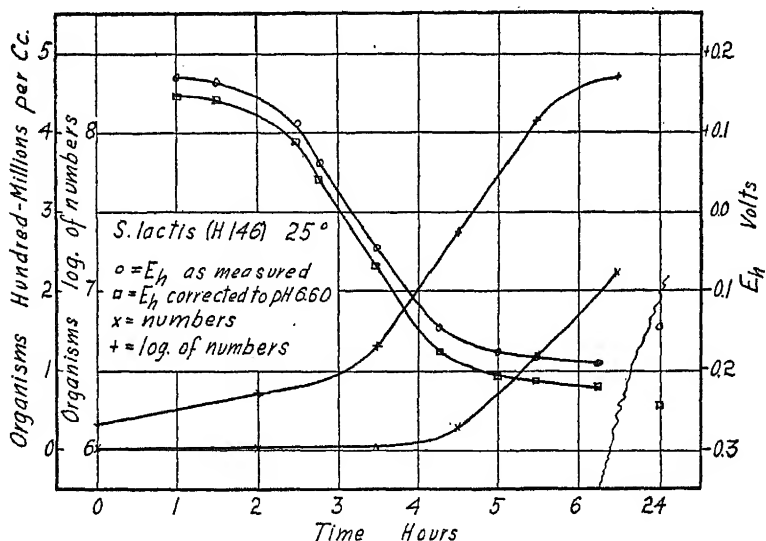


FIG. 1. NUMBERS AND REDUCTION POTENTIAL OF AN ACTIVE STRAIN OF STREPTOCOCCUS LACTIS IN MILK

in 50 per cent alcohol just alkaline to phenolphthalein. Direct counts were used instead of plate counts because of the long chains formed by a number of the organisms studied. Individual organisms were counted rather than groups.

Several preliminary experiments demonstrated that, for milk in the range of hydrogen-ion concentration from pH 4 to pH 7, the relationship between E_h and pH was practically a straight line function, E_h increasing by approximately 0.06 volt for each decrease of one pH unit at temperatures between 25° and 40°C. (Clark and Cohen, 1923).

This relationship has been used to recalculate all observed Eh values to the corresponding values at pH 6.6 for purposes of direct comparison. All plots of Eh are of values at pH 6.6 unless otherwise labelled.

At first, logarithms of the numbers of bacteria were used in plotting the growth curves, but actual numbers of bacteria have been plotted in the accompanying curves because numbers of cells seemed more significant than logarithms of these numbers. The

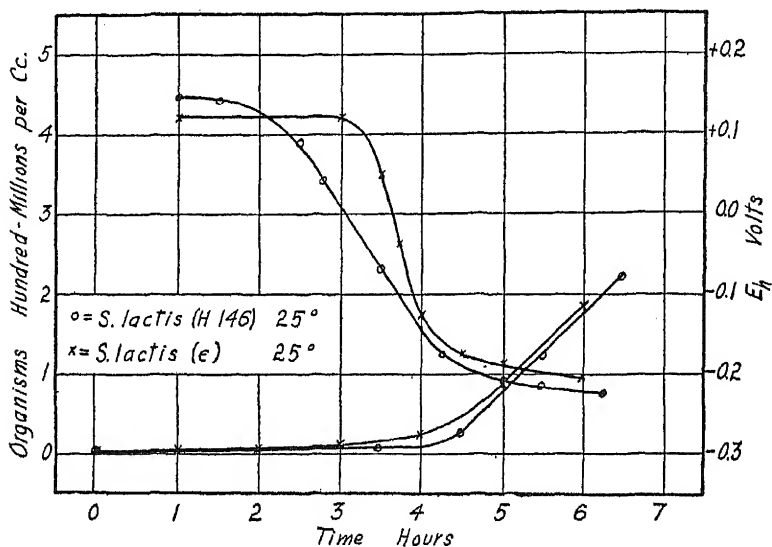


FIG. 2. NUMBERS AND REDUCTION POTENTIAL OF TWO STRAINS OF *STREPTOCOCCUS LACTIS*, EACH IN MILK AT 25°

difference in the resulting curves is shown by the growth curves of *Streptococcus lactis* at 25°C. in figure 1. It will be observed that the beginning of the rapid rise of the curve showing actual numbers is well within the logarithmic growth phase of the organism. In the same figure the curves of oxidation-reduction or Eh values are shown uncorrected for pH and corrected to pH 6.6.

EXPERIMENTAL

In figure 2 are shown results with two different strains of *S. lactis*, each at 25°C., and in figure 3 with two strains, each at

37°C. Strain "H 142" is a slow-growing one which produces less acid than the other strains. A longer time was needed to reach the bottom of its Eh curve and at six hours it had just reached the point of rapid increase in numbers. A comparison of strain "H 146" at 25° and at 37°C. shows that the lower temperature decreases the rate of growth and hence the rate of lowering of the Eh, but that about the same final Eh value is obtained at the time that an equal bacterial population is attained.

In this connection it is interesting to note that when the bottom of the Eh curve is reached, practically equal numbers of *S. lactis*

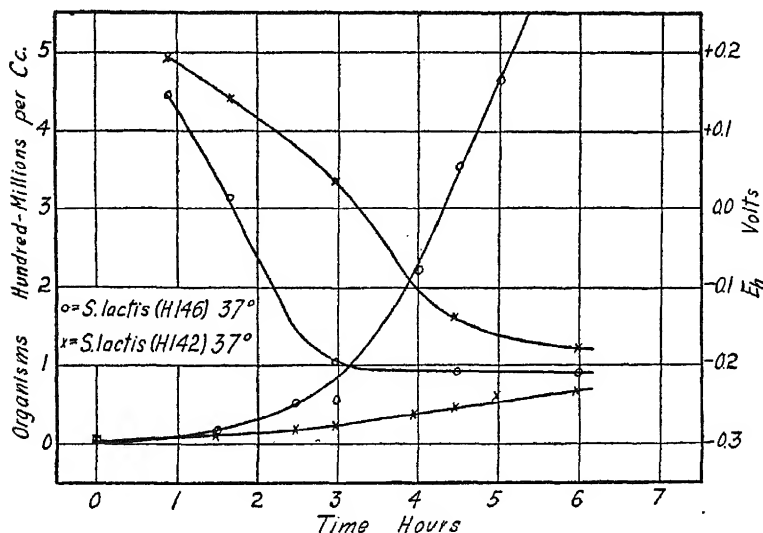


FIG. 3. NUMBERS AND REDUCTION POTENTIAL OF TWO STRAINS OF STREPTOCOCCUS LACTIS, EACH IN MILK AT 37°

are present with either of the more active strains, "E" or "H 146" and at either 25° or 37°C. Four different experiments with the "E" strain at 25°C. showed the following counts at the flattening of the Eh curve: 50 million, 50 million, 55 million and 57 million per cubic centimeter. The "H 146" strain at both 25° and 37°C. showed about 55 million organisms per cubic centimeter at the bottom of the Eh curve. With the weaker strain, "H 142," however, a greater number of organisms, 66 million, was necessary to produce a similar lowering of Eh.

Figure 2 also brings out a fact that will be noted in a number of the figures to follow: that the beginning of the rapid rise in the numbers of bacteria, as shown in the growth curve of actual counts, is almost coincident with the final flattening of the Eh curve, when the Eh values begin to drop very slowly, if at all. In the case of the two strains of *S. lactis* at 25°C. shown in figure 2, the Eh curve is flattening and the growth curve beginning to rise rapidly at about four and one-half hours from the time of

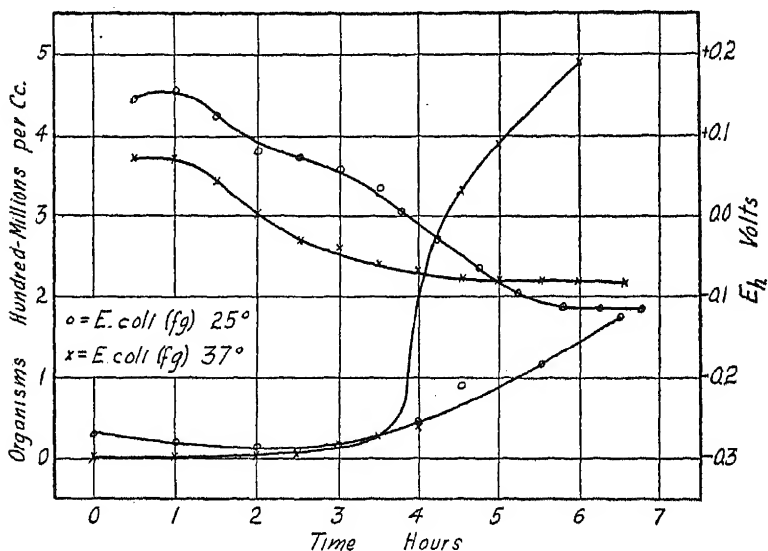


FIG. 4. NUMBERS AND REDUCTION POTENTIAL OF *ESCHERICHIA COLI* IN MILK AT 25° AND AT 37°

inoculation. The rapid drop in the Eh curve usually begins before the start of the phase of logarithmic growth of the organism, as is shown in the curves for *S. lactis* in figure 1.

The Eh curves of *S. lactis* usually show a period of fairly constant potential, followed by a sudden change to a negative value of about -0.20 . The curves then slowly continue in the same direction to a final value of -0.25 or -0.26 in the case of vigorous cultures. The time elapsing between inoculation and the beginning of the rapid change of potential is apparently determined

largely by the initial potential level (oxygen content) of the medium.

The curves of *Escherichia coli* at 37°C. again bring out the fact that the Eh curve starts to flatten at about the time that the growth curve begins to rise rapidly. Figure 4 shows Eh and growth curves of *E. coli* at 25° and 37°C. Potentiometric readings on *E. coli* were not always definite and could easily be disturbed by more than momentary pressure on the tap key when

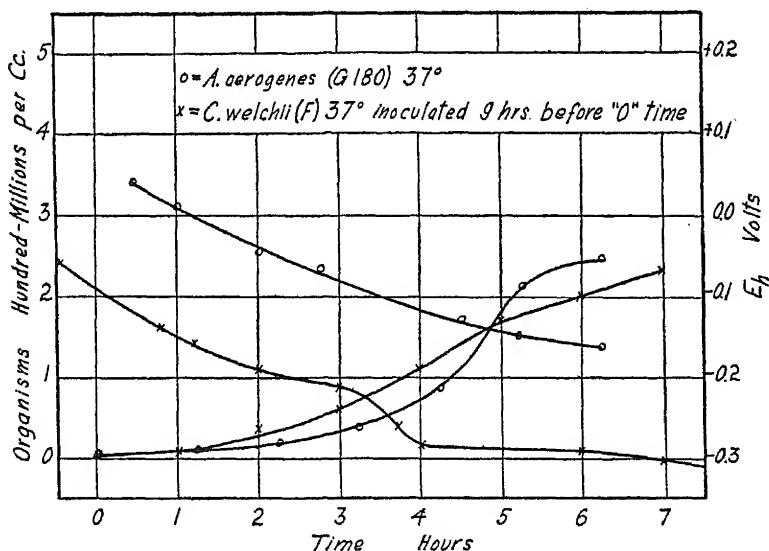


FIG. 5. NUMBERS AND REDUCTION POTENTIAL OF *AEROBACTER AEROGENES* AND OF *CLOSTRIDIUM WELCHII*, EACH IN MILK AT 37°

the opposing E.M.F.'s were not close to balance. Evidently, too, as might be expected, the culture was very sensitive to the introduction of air. By means of *E. coli*, it may be recalled, Cannan, Cohen and Clark (1926) were able to demonstrate the production of a hydrogen over-voltage. Our much more positive final value of -0.17 at pH 6.6 must be attributed to the extreme sensitiveness to air of the system set up by *E. coli*. To this same reason must be attributed the fact that the number of *E. coli* organisms at the bottom of the Eh curve was 37 million and 40 million in

two experiments, but 135 million and 145 million in two other experiments.

Figure 5 shows Eh and growth curves of *Aerobacter aerogenes* and *Clostridium Welchii* at 37°C. The *C. Welchii* culture had a long lag period and showed no increase in numbers until about the ninth hour after inoculation, which time is plotted as the zero hour in the curves in figure 5. A slow change in potential to a value only slightly less negative than that given by *S. lactis*

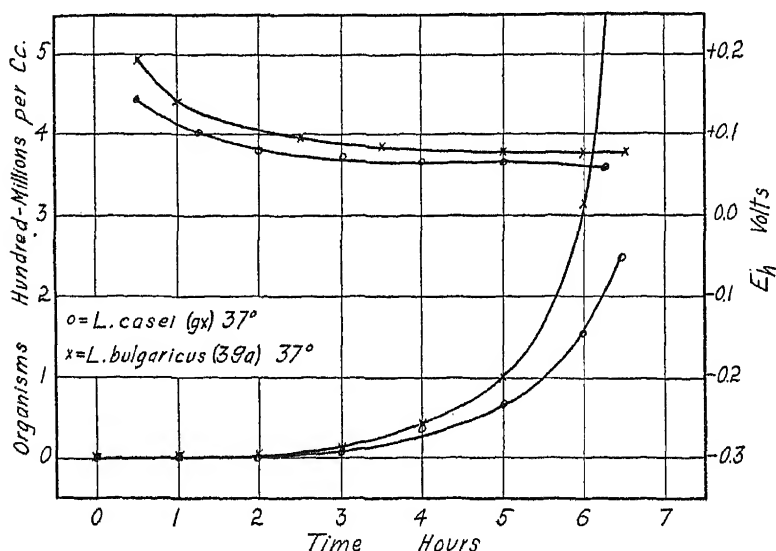


FIG. 6. NUMBERS AND REDUCTION POTENTIAL OF LACTOBACILLUS CASEI AND OF LACTOBACILLUS BULGARICUS, EACH IN MILK AT 37°

in milk is brought about by *A. aerogenes*. Bubbling air through cultures of this organism had a comparatively slight effect on the potential level. There were about 200 million of the organisms present when the Eh curve started to flatten, as compared to about 55 million of *S. lactis* at a corresponding position on the Eh curve, although almost the same Eh value was attained in both cases.

The characteristic potential curve for *C. Welchii* shows a fairly steady drop toward the most negative potential encountered in

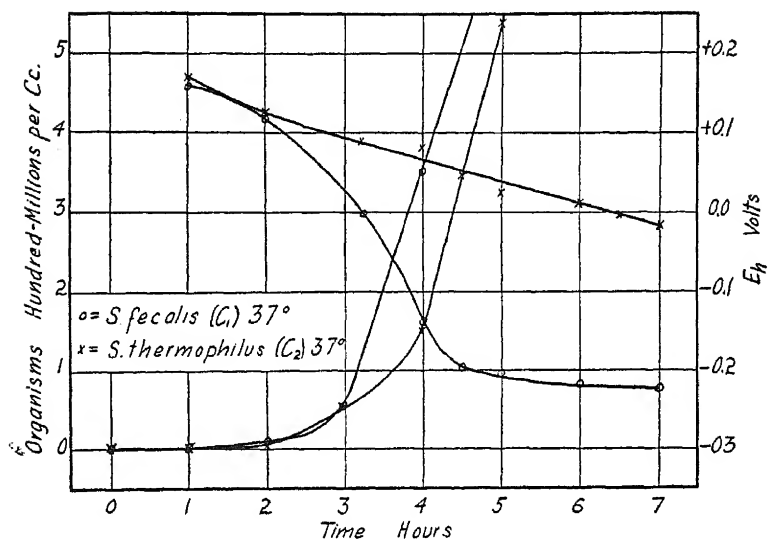


FIG. 7. NUMBERS AND REDUCTION POTENTIAL OF *STREPTOCOCCUS FECALIS* AND OF *STREPTOCOCCUS THERMOPHILUS*, EACH IN MILK AT 37°

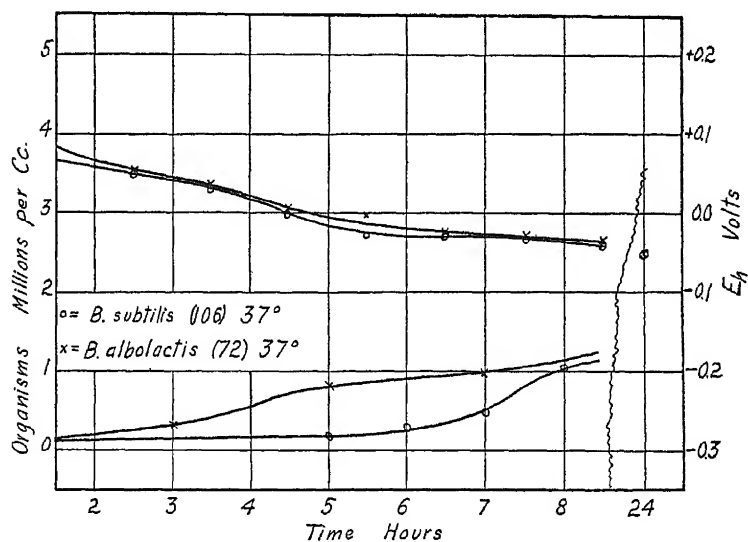


FIG. 8. NUMBERS AND REDUCTION POTENTIAL OF *BACILLUS SUBTILIS* AND OF *BACILLUS ALBOLACTIS*, EACH IN MILK AT 37°

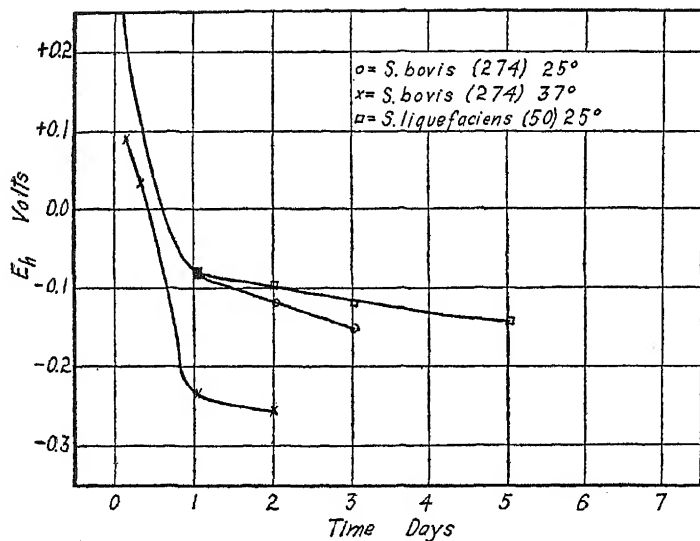


FIG. 9. REDUCTION POTENTIAL OF STREPTOCOCCUS BOVIS IN MILK AT 25° AND AT 37° , AND OF STREPTOCOCCUS LIQUEFACIENS IN MILK AT 25°

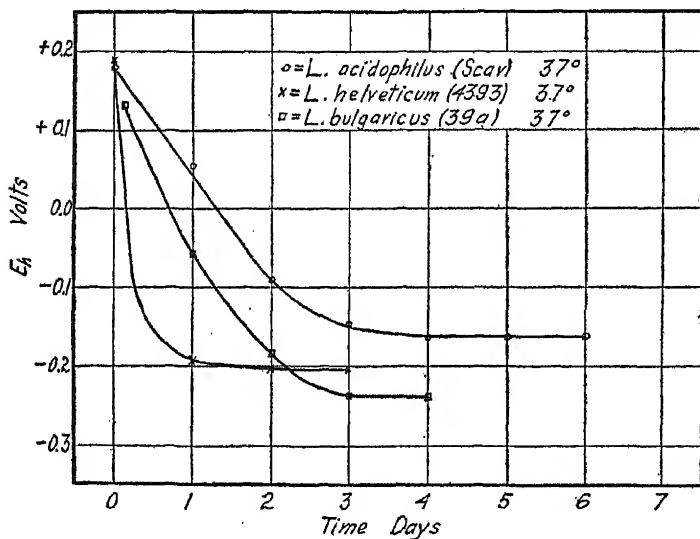


FIG. 10. REDUCTION POTENTIAL OF LACTOBACILLUS ACIDOPHILUS, OF LACTOBACILLUS HELVETICUM AND OF LACTOBACILLUS BULGARICUS, EACH IN MILK AT 37°

this work, -0.37 . This is a very close approach to the potential of the hydrogen electrode, a point worthy of note since no elaborate precautions were taken to exclude air.

Figure 6 shows Eh and growth curves of *Lactobacillus casei* and *Lactobacillus bulgaricus* at 37°C . The Eh curves for the two organisms are very similar. It usually takes several days, even at the optimum temperature of the bacteria, for their final Eh value of about -0.235 to be attained in milk. The Eh curve for *L. bulgaricus* over several days is shown in figure 10.

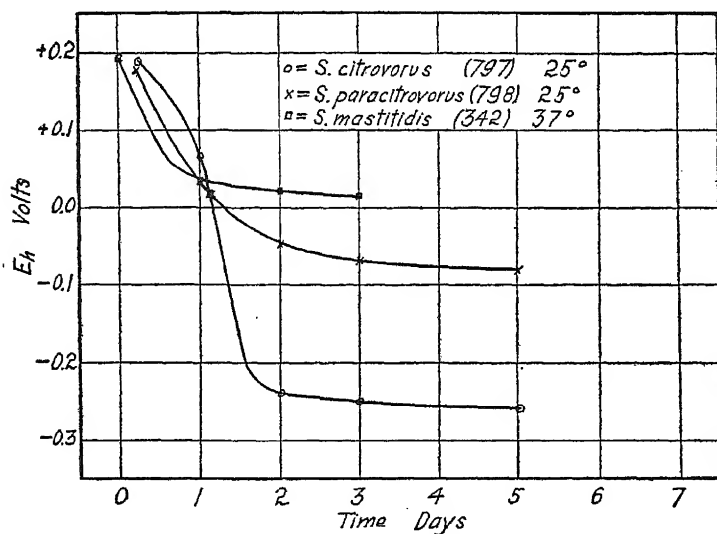


FIG. 11. REDUCTION POTENTIAL OF STREPTOCOCCUS CITROVORUS, AND OF STREPTOCOCCUS PARACITROVORUS EACH IN MILK AT 25° , AND OF STREPTOCOCCUS MASTITIDIS IN MILK AT 37°

Figure 7 shows Eh and growth curves of *Streptococcus fecalis* and *Streptococcus thermophilus* at 37°C . With both of these organisms it will be noted that considerable numbers of bacteria were present before the Eh curve had reached its bottom. Indeed, the Eh curve had only begun to drop when the growth curve started to rise. The Eh curve of *S. fecalis* approaches practically the same limit in milk as the curve of *S. lactis*, but the change is much more gradual. *S. thermophilus* gives a rather flat, straight curve to -0.14 volts at pH 6.6.

Bacillus subtilis and *Bacillus albolactis* (fig. 8) grew very slowly in milk at 37°C. and the Eh values showed a correspondingly slow and gradual drop. The final Eh value for both cultures after twenty-four hours, as shown to the right of the wavy line in figure 8, is very little below the value obtained at about six hours.

Figures 9, 10, and 11 show Eh curves for less common milk organisms. Time has been plotted in days. *Streptococcus bovis* at 37°C. (fig. 9), except for a slower production of acid, acts much like *S. lactis*. At 25°C. the action is still slower. *Streptococcus liquefaciens* (fig. 9) produces levels of potential more negative than those of *Streptococcus paracitrovorus* (fig. 11), but not as negative as those of *S. lactis*.

Potential levels of the order of -0.16 volt were slowly attained by *Lactobacillus acidophilus* (fig. 10). *Lactobacillus helveticum* (fig. 10) approached its final value of approximately -0.20 volt in about twenty-four hours. The value of -0.23 volt was reached by *Lactobacillus bulgaricus* in about three days.

The curve for *Streptococcus citrovorus* (fig. 11) closely resembles the *S. lactis* curve, while that of *S. paracitrovorus* approaches a limit about 0.15 volt more positive. *Streptococcus mastitidis* was not able to produce a negative potential in a period of three days.

SUMMARY

Changes in the oxidation-reduction potential of milk caused by various milk organisms in pure culture have been determined and plotted. Direct microscopic counts of the numbers of organisms present have been made on the milk and correlated with the changes in oxidation-reduction potential (Eh curves).

Under similar conditions each of the organisms studied produced changes in oxidation-reduction potential characteristic of that organism.

With many of the organisms studied, the end of the rapid drop in oxidation-reduction potential was almost coincident with the beginning of the rapid rise in numbers of bacteria. This point was well within the phase of logarithmic growth of the organism. With some of the organisms, however, like *S. fecalis*, *S. thermo-*

philus and *S. mastitidis* it was necessary to have present comparatively large numbers of actively growing organisms in order to bring the oxidation-reduction potential down toward its most negative value. These organisms might be said to produce a system with a weak quantity factor.

Experiments with two different strains of *S. lactis* at two different temperatures indicated that with vigorous cultures practically the same number of organisms was present at the end of the rapid drop in oxidation-reduction potential. A slower growing strain, which also produced less total acid, had more organisms present at a corresponding place on the Eh curve.

E. coli was found to bring about an oxidation-reduction system which was very easily thrown out of balance by such factors as the introduction of a little air or an unbalanced E.M.F. during potentiometric measurements. The system produced by *A. aerogenes*, on the other hand, showed practically no sensitiveness to air. The *S. lactis* system was not very sensitive to air, but the systems of the other streptococci were more sensitive, like that of *E. coli*.

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STUDIES ON THE INFLUENCE OF BACTERIA ON THE OXIDATION-REDUCTION POTENTIAL OF MILK

II. INFLUENCE OF ASSOCIATED CULTURES OF MILK ORGANISMS

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Received for publication October 11, 1930

In a previous paper (Frazier and Whittier, 1931) the influence of pure cultures of various milk bacteria on the oxidation-reduction potential of milk has been discussed. These results would be important in dealing with samples of milk in which one of these organisms predominated or in the extremely rare cases where practically a pure culture of one organism was present. In most samples of milk, however, two or more species of bacteria are present in considerable numbers and their associative growth may have an important effect on the multiplication of each and on the oxidation-reduction potential of the milk. In the work here reported, various pairs of cultures of milk bacteria have been inoculated in different proportions into milk, and changes in oxidation-reduction potential have been measured, as well as changes in numbers of bacteria of the two species.

METHODS

The methods for the measurement of Eh values and for the enumeration of bacteria were the same as those described in the first paper of this series (Frazier and Whittier, 1931). Reconstituted skim milk, sterilized by the intermittent method, was used as a medium and, unless otherwise specified, the inoculum was 1 cc. of an eighteen-hour milk culture of the organism. As in the previous paper, actual numbers of bacteria have been plotted instead of logarithms of numbers and all Eh values are at pH 6.6.

EXPERIMENTAL

In figures 1, 2 and 3 are shown results with *Streptococcus lactis* and *Escherichia coli* inoculated into milk in different proportions and incubated at 37°C. The incubation temperature is more favorable to *E. coli* than to *S. lactis*, and, consequently, with an approximately equal start the bacilli in most cases multiplied more rapidly at first than did the cocci, although the latter caught up after some hours.

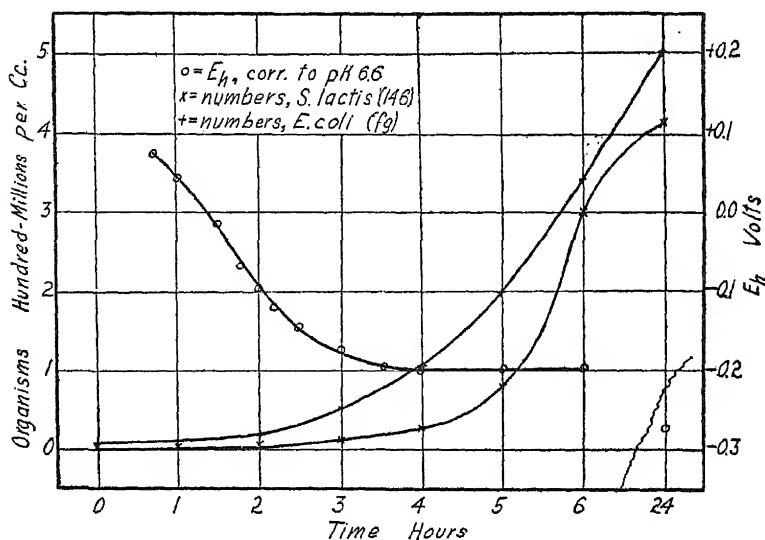


FIG. 1. NUMBERS AND REDUCTION POTENTIAL OF *STREPTOCOCCUS LACTIS* (0.5 CC. INOCULUM) AND *ESCHERICHIA COLI* (0.5 CC. INOCULUM) IN MILK AT 37°

When 0.5 cc. each of cultures of *S. lactis* and *E. coli* were added to milk (fig. 1), both cultures grew well and the Eh curve was more like that of *S. lactis* than that of *E. coli*, although the curve flattened at about -0.2 volt instead of going down to -0.22, the figure characteristic for this strain of *S. lactis*. The Eh curve flattened before the beginning of rapid rise in the growth curve of *S. lactis* and after rapid rise had started in the *E. coli* curve. In this case, then, *S. lactis* predominated in its influence on the oxidation-reduction potential, but *E. coli* had a slight restraining influence.

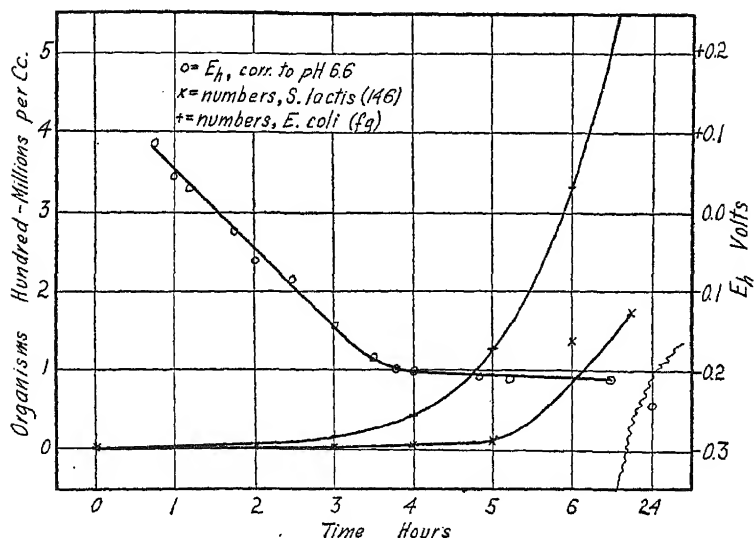


FIG. 2. NUMBERS AND REDUCTION POTENTIAL OF *STREPTOCOCCUS LACTIS* (1.0 CC. INOCULUM) AND *ESCHERICHIA COLI* (0.1 CC. INOCULUM) IN MILK AT 37°

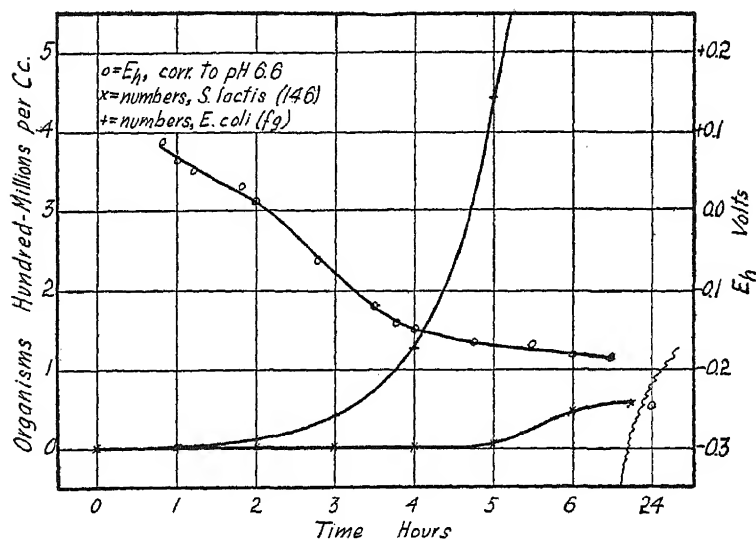


FIG. 3. NUMBERS AND REDUCTION POTENTIAL OF *STREPTOCOCCUS LACTIS* (0.1 CC. INOCULUM) AND *ESCHERICHIA COLI* (1.0 CC. INOCULUM) IN MILK AT 37°

When the inoculum was 1.0 cc. of *S. lactis* culture and 0.1 cc. of *E. coli* culture (fig. 2), the Eh curve had the characteristics of an *S. lactis* curve, although the *E. coli* organisms had apparently delayed the drop in Eh to some extent.

When 1.0 cc. of *E. coli* culture and 0.1 cc. of *S. lactis* culture were added (fig. 3), the Eh curve was more that of *E. coli*, although after six hours the Eh values were still dropping toward values characteristic of *S. lactis*.

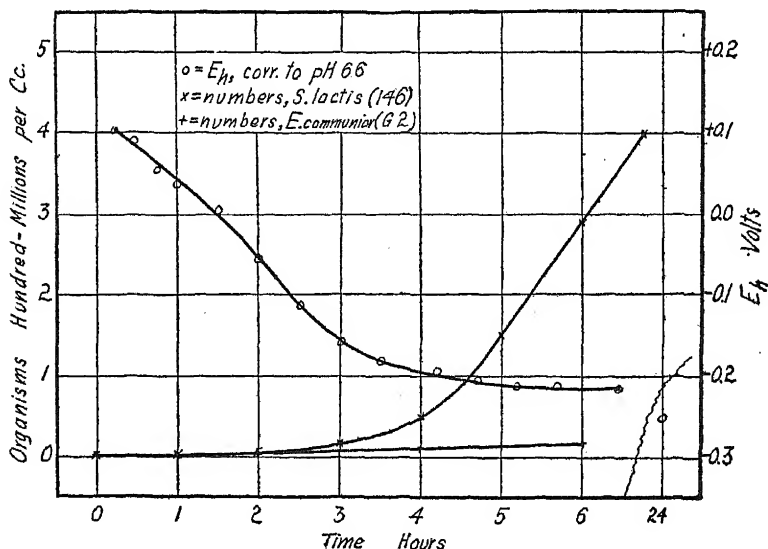


FIG. 4. NUMBERS AND REDUCTION POTENTIAL OF *STREPTOCOCCUS LACTIS* (1.0 CC. INOCULUM) AND *ESCHERICHIA COMMUNIOR* (1.0 CC. INOCULUM) IN MILK AT 37°

In all three figures the Eh value after twenty-four hours is that usually given by *S. lactis*, about -0.25 volt at pH 6.6. In a mixture of *S. lactis* and *E. coli*, then, the rod tends to hold back the drop in oxidation-reduction potential, but the coccus determines the final oxidation-reduction potential and from the start influences it more strongly than does the rod.

Figures 4 and 5 show results when *S. lactis* and *Escherichia communior* were grown together. When one cubic centimeter of each of the organisms was inoculated into milk, and the milk

was incubated at 37°C. (fig. 4), the Eh curve was like that of *S. lactis*, although the drop in Eh values was a little slower than with a pure culture of *S. lactis*. When the inoculum was 2.0 cc. of *E. communior* and 0.5 cc. of *S. lactis* (fig. 5) and the rod grew well from the start, the Eh values dropped very slowly over a period of about nine hours, and not until the *S. lactis* organisms started to increase at a rapid rate after ten hours did the Eh curve drop sharply. This strain of *E. communior* seemed to have an even more powerful restraining action than *E. coli* on the reducing

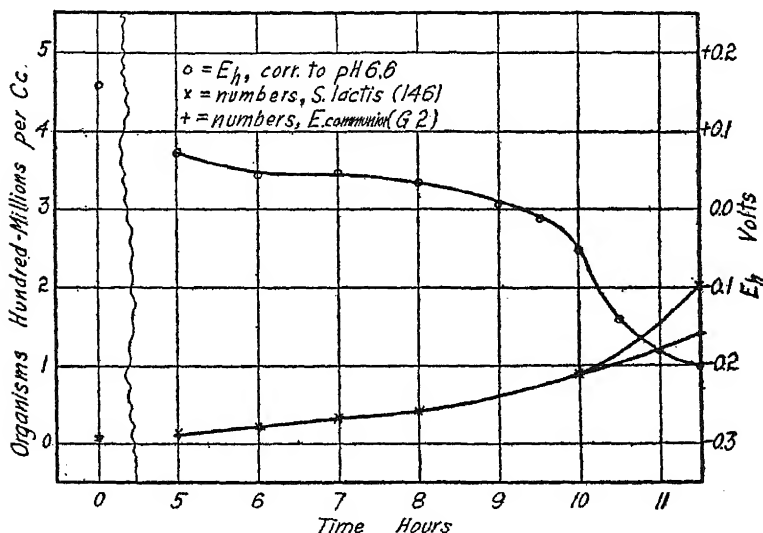


FIG. 5. NUMBERS AND REDUCTION POTENTIAL OF STREPTOCOCCUS LACTIS (0.05 CC. INOCULUM) AND ESCHERICHIA COMMUNIOR (2.0 CC. INOCULUM) IN MILK AT 37°

activity of *S. lactis*. About 200 million *S. lactis* cells were present when the Eh value had dropped to -0.2 volt. In the previous paper it was noted that the same value was attained in pure culture when only about 55 million of this same strain of *S. lactis* were present.

When an inoculum of one cubic centimeter each of *S. lactis* and *Aerobacter aerogenes* was used (fig. 6), the coccus predominated and controlled the changes in oxidation-reduction potential.

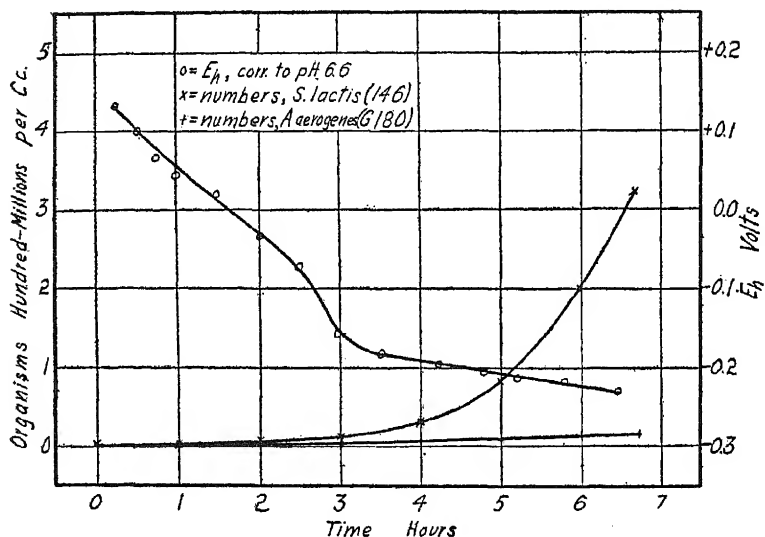


FIG. 6. NUMBERS AND REDUCTION POTENTIAL OF *STREPTOCOCCUS LACTIS* (1.0 CC. INOCULUM) AND *AEROBACTER AEROGENES* (1.0 CC. INOCULUM) IN MILK AT 37°

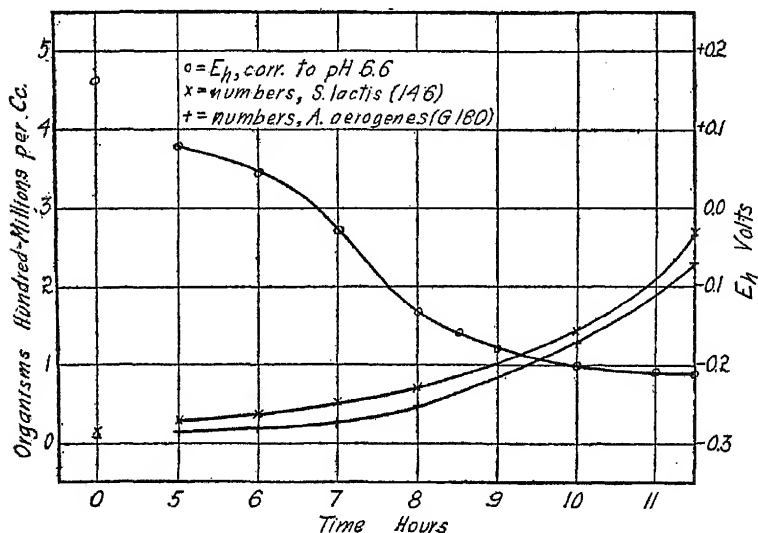


FIG. 7. NUMBERS AND REDUCTION POTENTIAL OF *STREPTOCOCCUS LACTIS* (0.05 CC. INOCULUM) AND *AEROBACTER AEROGENES* (2.0 CC. INOCULUM) IN MILK AT 37°

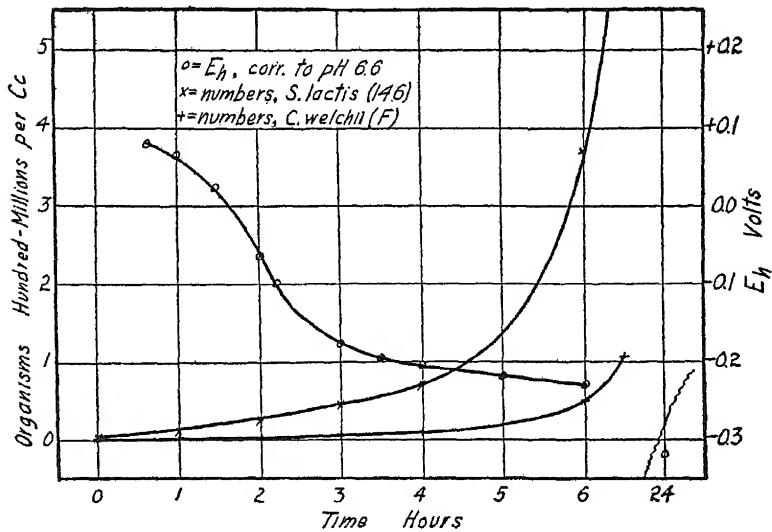


FIG. 8. NUMBERS AND REDUCTION POTENTIAL OF *STREPTOCOCCUS LACTIS* (1.0 CC. INOCULUM) AND *CLOSTRIDIUM WELCHII* (1.0 CC. INOCULUM) IN MILK AT 37°

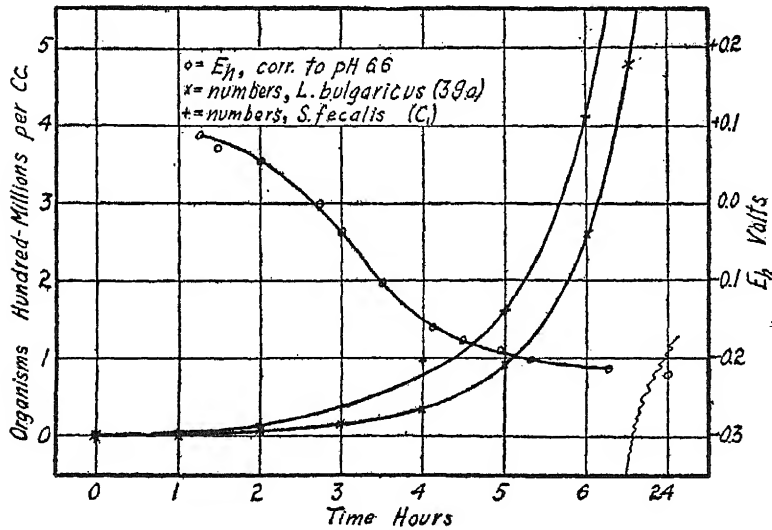


FIG. 9. NUMBERS AND REDUCTION POTENTIAL OF *LACTOBACILLUS BULGARICUS* (1.0 CC. INOCULUM) AND *STREPTOCOCCUS FECALIS* (1.0 CC. INOCULUM) IN MILK AT 37°

When 2.0 cc. of *A. aerogenes* and 0.05 cc. of *S. lactis* were added (fig. 7) and both organisms obtained a good start, the drop in the Eh curve was delayed for several hours, and the number of *S. lactis* organisms present when the Eh value reached -0.2 volt was about 150 millions, instead of about 50 millions when *S. lactis* predominated (fig. 6).

From an examination of the Eh curves for pure cultures of *S. lactis*₂^W and of colon-aerogenes organisms (paper I of this series),

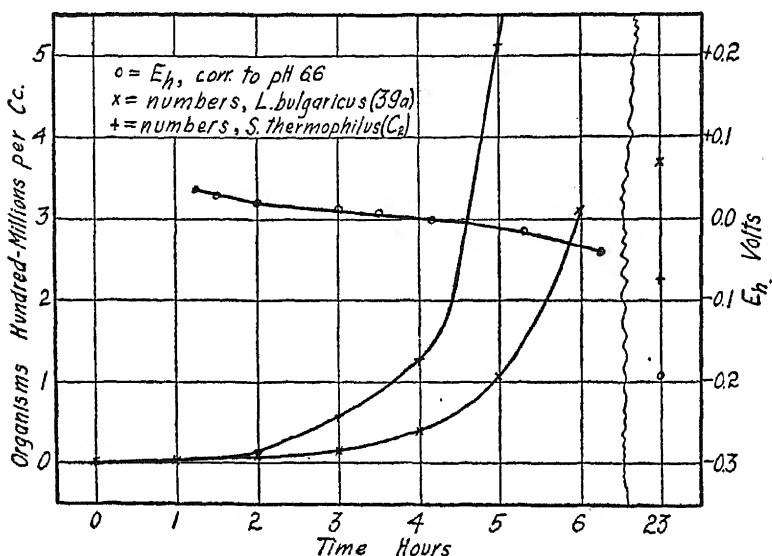


FIG. 10. NUMBERS AND REDUCTION POTENTIAL OF LACTOBACILLUS BULGARICUS (1.0 CC. INOCULUM) AND STREPTOCOCCUS THERMOPHILUS (1.0 CC. INOCULUM) IN MILK AT 37°

it would seem probable that the combination of lactic organism and gas-former would hasten the drop in Eh to the value at which the Eh curve of a pure culture of the gas-former begins to flatten. From this point on the colon-aerogenes organism would tend to retard a further drop in oxidation-reduction potential to the value characteristic of *S. lactis*. Therefore, a combination of *S. lactis* and a colon-aerogenes organism would tend to cause a more rapid methylene blue reduction than would *S. lactis* alone.

Under the conditions of the experiment the anaerobic *Clostridium Welchii* did not get well started when added to milk with *S. lactis* (fig. 8), and the Eh curve for the first five or six hours had the characteristics of a *S. lactis* curve. The anaerobe did, however, cause the Eh value to drop down to about -0.32 volt in twenty-four hours.

In figure 9 *Streptococcus fecalis* apparently completely controlled the Eh changes when it was grown with *Lactobacillus bulgaricus*, although both organisms grew well. In the same way *Streptococcus thermophilus* dominated in Eh changes when grown with *L. bulgaricus* (fig. 10). These three organisms are important in Swiss cheese and often grow together there.

The work of Marshall and Farrand (1908) and others on the associative action of *Bacillus subtilis* and *S. lactis* has shown that *S. lactis* is helped by the combination. Experiments with *B. subtilis* and *S. lactis* and with *Bacillus albolactis* and *S. lactis* showed that the proteolytic rods had little influence on changes in Eh and the curves were as if *S. lactis* alone were present.

SUMMARY

Pairs of species of milk bacteria were grown together in sterile milk at 37°C . Changes in oxidation-reduction potential were measured and compared with changes in the numbers of bacteria of the two species.

E. coli, *E. communior* and *A. aerogenes*, when grown with *S. lactis*, all exerted a restraining influence on the rapid drop in Eh values usually caused by pure cultures of *S. lactis*. The larger the proportion of actively growing colon-aerogenes organisms, the greater was the restraining action. When growing with one of the colon-aerogenes organisms, apparently more *S. lactis* organisms must be present to cause a drop in Eh values to -0.2 volt than when the *S. lactis* organisms are in pure culture.

When *C. Welchii* was grown with *S. lactis* in milk, the lactic organism controlled Eh changes during the first part of the growth, but *C. Welchii* brought about a final negative value of about -0.32 volt.

S. fecalis and *S. thermophilus* each controlled the changes in Eh when grown with *L. bulgaricus* in milk.

B. subtilis and *B. albolactis* did not materially affect the Eh changes when grown with *S. lactis* in milk.

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STUDIES ON THE PROTEOLYTIC BACTERIA OF MILK

V. ACTION OF PROTEOLYTIC BACTERIA ON MILK SERUM

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Received for publication October 11, 1930

In previous papers of this series (Frazier and Rupp, 1928), the action of proteolytic bacteria of milk on casein and on skim milk has been reported. In order to make the study of proteolysis in milk more complete a study was made of the action of these organisms on milk from which the casein and lactoglobulin had been removed and in which lactalbumin was the only remaining protein. A number of the proteolytic bacteria were found to cause an increase in ammonia and in amino-nitrogen according to the formol titration method in milk, but were unable to split casein in any of the media used. This led to the supposition that lactalbumin might have been decomposed. In this work the organisms were grown in a milk serum, made by the removal of all casein and lactoglobulin from milk. This milk serum contained lactalbumin as its only protein and small amounts of non-protein nitrogen.

No references have been found to the action of bacteria on milk serum, although Franssen (1929) recommends its use in culture media. The only reference to the action of bacteria on lactalbumin is a preliminary report by Supplee (1917) on the action of milk bacteria on some of the nitrogenous constituents of milk. He obtained results which suggested that lactalbumin might have been decomposed. He grew organisms in milk for twenty-four hours at 30°C. and then analyzed for casein, albumin and other fractions. He sometimes found an increase over the control in the albumin fraction and concluded that the increase was due to proteoses from the casein. In a few cases, however,

he found a decrease in the albumin fraction and concluded that some species of bacteria could attack lactalbumin more readily than casein. Among the organisms which caused a decrease in the albumin were: *Mic. albidus*, *Ps. liquefaciens*, *Bact. bulgaricum*, *B. coli-communis* and *Bact. aerogenes*.

METHODS

The milk serum used in these experiments was obtained by ultrafiltration of skim milk through a collodion membrane. In this way all of the casein, and probably all of the lactoglobulin, were removed. The serum was sterilized by filtration through a Berkefeld filter candle. All flasks of serum were incubated for several days at 30°C. to test for sterility.

Tests on the milk serum showed the absence of casein. If traces of casein had remained in the serum after ultrafiltration, they would have been removed by the Berkefeld filter. All tests for lactoglobulin in the milk serum were either clearly negative or doubtful. Negative results were obtained by half-saturation of the serum with ammonium sulphate, by saturation with sodium chloride with slight acidification, and by the method of Howe (1922) for the precipitation of globulin from milk serum. Howe found that 21.5 per cent of sodium sulphate would precipitate the euglobulin and two pseudo-globulins and leave the albumin in solution. No trace of a precipitate was obtained with 21.5 per cent sodium sulphate in the milk serum used in these experiments.

Analyses of the serum for total nitrogen showed between 0.06 and 0.066 per cent in different tests, or, expressed as lactalbumin, between 0.38 and 0.422 per cent. The Berkefeld candle removed very little nitrogen. According to Denis and Minot (1919) the average amount of non-protein nitrogen in milk is about 21 mgm. in 100 cc., of which 9 to 10 mgm. is urea and the remainder chiefly amino-acid, creatine and creatinine nitrogen. Bleyer and Kallmann (1924) found about the same amount of urea. Analyses of the milk serum used in these experiments showed about 25 to 28 mgm. of non-protein nitrogen per 100 cc. of serum or 23 to 26 mgm. per 100 cc. of milk.

The milk serum cultures of the proteolytic bacteria were ana-

lyzed for total, protein, non-protein, amino and ammonia nitrogen. Protein (lactalbumin) nitrogen was estimated by difference between total nitrogen and non-protein nitrogen. Cultures of the organisms in skim milk were analyzed for casein nitrogen as well as total, non-protein, amino and ammonia nitrogen.

Total nitrogen was determined by the Kjeldahl method: 3.0 grams of milk or milk serum were used with 5.0 grams of sodium sulphate, 0.1 gram of copper sulphate and 15 cc. of concentrated sulphuric acid.

Casein nitrogen in the milk was determined by precipitation with 10 per cent acetic acid and analysis of the precipitate for nitrogen. Precipitation with a saturated solution of alum was also used (Van Slyke and Hart, 1902). Alum is supposed to precipitate the caseoses also.

In the determination of non-protein nitrogen 10 cc. of milk or milk serum were treated with 70 cc. of water, 10 cc. of 10 per cent sodium tungstate solution and 10 cc. of sulphuric acid (Folin and Wu, 1919). The filtrate was analyzed for nitrogen.

Ammonia nitrogen was determined by the Folin aeration method (Hawk, 1923) in 50 cc. of the non-protein nitrogen filtrate.

Amino nitrogen was determined on 5 cc. of the ammonia-free filtrate by the Folin (1922) colorimetric method and also by the Sörensen formol titration method on 10 cc. of the milk or milk serum in 35 cc. of water. This formol titration figure includes ammonia, and therefore the amount of ammonia nitrogen previously found was subtracted to obtain the amino nitrogen. Titratable acidity was found by titration with N/10 sodium hydroxide to a pink color with phenolphthalein.

RESULTS

Tables 1 and 2 show the results of analyses of cultures of proteolytic bacteria in milk serum after incubation for ten days at 30°C. For the purpose of comparison, analyses of cultures of *S. lactis*, *L. bulgaricus*, *A. aerogenes*, and *L. casei* in milk serum have been included in table 1. The figures given in the tables were obtained by subtracting the control, and gains over the

control are indicated by positive quantities and losses by negative quantities. Ability of the organisms to ferment urea has also been included in the tables.

Most of the cocci (table 1) show a comparatively small decrease in protein nitrogen with a correspondingly small increase in non-

TABLE 1
Action of proteolytic cocci on milk serum (also four lactic acid bacteria)

ORGANISM	pH	TITRAT- ABLE ACID- ITY*	NON- PRO- TEIN NITRO- GEN†	PRO- TEIN NITRO- GEN	FREE AMMO- NIA	AMINO- NITRO- GEN (FOLIN)	AMINO NITRO- GEN (SOREN- SON)	UREA FERMENTA- TION
		cc.	mgm.	mgm.	mgm.	mgm.	mgm.	
Control.....	6.7	0.0	0.0	0.0	0.0	0.0	0.0	
<i>M. citreus</i>	6.0	+7.8	+4.5	-4.5	+1.5	+1.5	-0.5	-
<i>M. perflavus</i>	6.5	-3.3	+2.4	-2.4	+11.7	+1.0	-2.9	+
<i>M. varians</i>	6.3	+6.9	+2.8	-2.8	+12.3	+1.0	-0.7	+
<i>M. casei</i> (yellow).....	6.2	+6.0	+5.6	-5.6	+1.5	+2.5	-1.5	-
<i>M. percitreus</i>	6.7	+1.0	-14.7	+14.7	+2.9	+1.0	-0.4	+
<i>M. cereus</i>	6.7	-3.3	+2.2	-2.2	+1.8	+1.0	+2.2	-
<i>M. subflavescens</i>	6.8	-1.0	+7.8	-7.8	+8.1	0.0	+1.2	-
<i>M. luteus</i>	6.4	+1.8	+20.3	-20.3	+12.7	+2.0	+0.5	+
P 147.....	6.1	-1.9	+7.4	-7.4	+7.4	0.0	0.0	+
<i>M. casei</i> (white).....	5.9	+9.8	+2.3	-2.3	+1.0	+2.0	+0.1	-
<i>Staph. albus</i>	5.8	+11.7	+2.6	-2.6	+0.5	+2.0	+1.8	-
<i>M. freudenreichii</i>	6.0	+5.6	+0.4	-1.1	+13.0	+2.0	-1.7	+
<i>M. ureae</i>	8.0	-7.4	-3.6	+3.6	+16.1	0.0	-3.7	+
P 204.....	6.1	+8.0	-2.4	+2.4	-0.3	0.0	+1.4	-
P 269.....	4.7	+1.5	-12.0	+12.0	+13.4	-2.0	0.0	+
<i>Str. liquefaciens</i>	4.8	+12.8	+0.7	-2.1	+0.3	+1.0	0.0	-
<i>Str. bovis</i>	5.6	+9.1	+0.7	-0.7	+8.0	+1.0	+5.3	-
<i>Str. lactis</i>	4.3	+24.3	+6.8	-6.8	-1.1	+4.0	+0.1	-
<i>L. bulgaricus</i>	3.5	+11.4	+7.4	-7.4	+1.5	+2.0	-1.1	-
<i>A. aerogenes</i>	4.9	+19.6	-5.6	+5.6	+6.5	+2.0	-1.1	+
<i>L. casei</i>	3.4	+13.4	+6.2	-6.2	+4.1	+4.0	-3.0	-

* Increase or decrease in acidity expressed as cubic centimeters of N/10 NaOH per 100 cc. of medium.

† All nitrogen figures expressed as increase or decrease in milligrams per 100 cc. of medium.

protein nitrogen. This would indicate a small amount of decomposition of lactalbumin. With bacteria like *M. citreus* and *M. casei*, most of this gain in non-protein nitrogen can be accounted

for as ammonia and amino-nitrogen. Such cocci as *M. perflavus* and *M. varians*, however, produce a much larger amount of

TABLE 2
Action of proteolytic rods on milk serum

ORGANISM	pH	TITRAT- ABLE ACID- ITY*	NON- PRO- TEIN NITRO- GEN†	PRO- TEIN NITRO- GEN	FREE AMMO- NIA	AMINO- NITRO- GEN (FOLIN)	AMINO- NITRO- GEN (SOREN- SON)	UREA FERMENTA- TION
		cc.	mgm.	mgm.	mgm.	mgm.	mgm.	
Control.....	6.7	0.0	0.0	0.0	0.0	0.0	0.0	
<i>Flavobacterium</i>								
<i>synxanthum</i>	8.1	-2.2	+13.7	-13.7	+25.3	+6.0	+12.2	-
<i>Flavobacterium lactis</i>	8.2	-10.6	+13.1	-13.1	+18.6	-0.5	-3.4	-
<i>Flavobacterium</i>								
<i>tremelloides</i>	6.3	+3.8	+10.4	-10.4	+6.0	+1.5	-0.4	-
<i>Serratia marcescens</i>	8.1	-5.2	+2.3	-2.3	+6.4	-1.0	0.0	+
P 268 (<i>Serratia</i>).....	8.2	-11.9	-5.7	+5.7	+9.0	0.0	-2.7	+
<i>Achromobacter</i>								
<i>coadunatum</i>	5.5	+8.2	-4.6	+4.2	+0.2	+1.0	-0.2	+(acid)
<i>Achromobacter</i>								
<i>liquefaciens</i>	7.0	-5.0	+11.7	-11.7	+9.7	0.0	-2.3	-
<i>Achromobacter</i>								
<i>delicatulum</i>	7.7	+6.2	-13.1	+13.1	+2.7	0.0	-0.6	+(acid)
<i>Alcaligenes bookeri</i>	8.2	-8.9	+8.7	-9.9	+10.0	-1.5	-2.0	-
<i>Proteus vulgaris</i>	7.1	-1.1	-10.6	+10.6	+2.4	-2.0	-1.7	+(acid)
P 107 (<i>Escherichia</i>).....	5.0	+32.5	-5.1	+5.2	+13.1	0.0	0.0	+
<i>B. albolactis</i>	5.9	+13.8	+4.7	-4.7	+0.4	+3.5	-1.0	-
<i>B. cereus</i> "A".....	8.4	-13.0	+16.9	-16.9	+7.1	+1.7	-4.1	-
<i>B. cereus</i> "B".....	8.0	-12.2	+11.1	-11.1	+6.9	+1.0	-4.6	-
<i>B. vulgatus</i>	8.4	-11.4	+17.3	-17.3	+6.2	+4.0	-4.5	-
<i>B. subtilis</i>	8.0	-9.9	+13.9	-13.9	+8.0	0.0	+0.6	+
<i>B. simplex</i>	7.7	-0.2	+15.9	-15.9	+16.8	+2.0	0.0	+
<i>B. mesentericus</i>	6.8	-2.0	-1.0	+1.0	+1.8	0.0	0.0	-
<i>B. cohaerens</i>	6.7	-4.4	+4.9	-4.9	+0.6	-1.0	+3.0	-
<i>B. tumescens</i>	8.4	-17.9	+4.1	-4.1	+10.7	-2.0	+0.5	+
<i>B. megatherium</i>	8.4	-15.1	+18.7	-18.7	+7.3	0.0	-1.1	-
<i>B. ruminatus</i>	7.2	+0.7	+12.2	-12.2	+12.9	+1.5	+5.2	+
<i>B. macerans</i>	5.8	+7.8	+10.9	-10.9	+0.6	+2.0	+3.6	-
P 67.....	8.6	-13.9	-7.8	+7.8	+5.1	-2.0	0.0	+
P 285.....	6.4	+3.1	-6.4	+6.4	+2.7	0.0	0.0	-

* See footnote to table 1.

† See footnote to table 1.

ammonia nitrogen than can be accounted for by loss of protein nitrogen. These organisms are able to break down urea, and the

amount of ammonia found corresponds closely to the amount which would be formed from 10 to 12 mgm. of urea nitrogen in the milk serum (Denis and Minot, 1919).

It is difficult to interpret the results obtained with *M. percitreus*, *M. ureae*, P 204 and P 269, where a large increase in protein nitrogen was obtained with the analytical methods used and a decrease in non-protein nitrogen. *M. percitreus* is apparently able to decompose lactalbumin with an increase in amino-nitrogen. In one analysis there was an increase of as much as 6.2 mgm. of amino-nitrogen per 100 cc. of milk serum. This organism is also very actively caseolytic. *M. ureae* and P 269, like *M. percitreus*, are able to decompose urea, but unlike *M. percitreus* cause a considerable increase in ammonia nitrogen. Their action in this way resembles that of the urea fermenters *perflavus* and *M. varians*.

The Gram-negative rods (table 2), like the cocci, may be divided into those which decrease protein nitrogen and increase non-protein nitrogen and those which accomplish the opposite result. Organisms like the *Flavobacteria*, *Achromobacter liquefaciens* and *Alcaligenes bookeri* are obviously able to decompose the lactalbumin in milk serum. *Achromobacter coadunatum* would undoubtedly be able to break down lactalbumin in the absence of fermentable sugar as it did casein (Paper III of this series).

All of the Gram-positive, spore-forming rods except *B. mesentericus* decomposed the lactalbumin. In most cases there was a marked increase in ammonia. It will be observed that, even with these actively proteolytic bacteria, there was not a large gain in amino-nitrogen.

In agreement with the results of Supplee (1917) *L. bulgaricus* in table 1 is apparently shown to break down lactalbumin to some extent although *A. aerogenes* did not do so in these experiments. Both *L. casei* and *S. lactis* also increased the non-protein nitrogen and decreased the protein nitrogen.

In order to compare the action of the proteolytic bacteria on milk and on milk serum (milk minus casein and lactoglobulin) table 3 has been given in addition to tables 1 and 2. Table 3 shows the results of quantitative analyses of ten-day milk cultures for the

various nitrogen fractions. It will be observed that a number of the cocci, which break down large amounts of casein in milk, do not decrease the amount of protein in milk serum to a great

TABLE 3
Action of proteolytic bacteria on milk (also two lactic acid bacteria)

ORGANISM	pH	TITRAT- ABLE ACID- ITY*	CASEIN NITRO- GEN (ACETIC)	CASEIN NITRO- GEN (ALUM)	NON- PROTEIN NITRO- GEN	FREE AMMO- NIA	AMINO- NITRO- GEN (FOLIN)	AMINO- NITRO- GEN (SOREN- SON)
		cc.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
Control.....	6.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>M. citreus</i>	5.6	+26.7	-209.4	-199.2	+69.3	+0.7	+6.0	+5.1
<i>M. perflavus</i>	5.9	+29.4	-162.3	-175.3	+110.0	+14.3	0.0	+2.2
<i>M. varians</i>	5.4	+36.0	-270.1	-254.2	+65.8	+12.3	0.0	+1.2
<i>M. casei</i> (yellow).....	5.7	+35.4	-265.1	-253.9	+115.2	+2.4	0.0	+10.5
<i>M. percitreus</i>	5.3	+24.8	-158.4	-160.8	+50.1	+5.2	+2.0	+1.1
<i>M. cereus</i>	6.5	-0.9	-10.0	+1.9	+6.7	+0.2	+2.0	-1.4
<i>M. luteus</i>	5.4	+28.4	-399.6	-383.0	+245.8	+7.2	+10.0	+13.6
<i>M. casei</i> (white).....	5.7	+31.9	-65.7	-88.7	+35.0	+1.1	+2.0	+1.7
<i>Staph. albus</i>	5.8	+35.8	-230.0	-221.3	+68.6	+2.7	-4.0	+7.3
<i>M. freudenreichii</i>	5.6	+35.3	-38.6	-37.1	+36.0	+10.1	+0.9	+2.1
<i>Str. liquefaciens</i>	4.8	+98.3	-302.9	-311.8	+180.4	+1.7	+6.0	+8.2
<i>Str. bovis</i>	5.6	+25.1	-292.0	-293.3	+90.0	+1.4	-2.0	+7.6
<i>Flavobacter synxanthum</i> .	7.0	+26.4	-415.9	-411.7	+359.0	+42.0	+90.0	+45.1
<i>Achromobacter</i> <i>coadunatum</i>	5.8	+38.5	-54.5	-58.8	+10.1	+0.4	0.0	-0.5
<i>Achromobacter</i> <i>liquefaciens</i>	6.8	+15.7	-177.9	-176.2	+146.8	+20.9	+26.0	+17.5
<i>Alcaligenes bookeri</i>	8.2	+12.9	-372.4	-358.9	+343.7	+43.0	+110.0	+86.6
<i>Proteus vulgaris</i>	6.8	+25.3	-265.3	-260.4	+218.6	+26.1	+30.0	+22.6
<i>B. albolactis</i>	5.3	+68.7	-245.3	-243.9	+232.5	+8.6	+13.5	+12.1
<i>B. cereus</i> "A".....	7.3	+19.4	-333.2	-319.7	+300.1	+27.2	+26.0	+15.0
<i>B. cereus</i> "B".....	7.3	+10.2	-364.7	-344.3	+333.7	+45.0	+39.0	+31.4
<i>B. vulgatus</i>	6.9	+34.9	-427.4	-425.2	+370.9	+18.5	+18.0	+13.8
<i>B. subtilis</i>	7.4	+22.3	-425.8	-385.3	+409.4	+6.5	+24.0	+17.2
<i>B. simplex</i>	6.5	+9.2	-185.4	-174.6	+101.8	+4.2	+1.0	+7.1
<i>B. cohaerens</i>	6.8	+11.8	-259.4	-218.7	+130.5	+7.3	+11.0	+10.5
<i>L. bulgaricus</i>	3.5	+203.8	-11.7	-19.7	+9.8	-0.3	-2.0	-3.5
<i>L. casei</i>	3.5	+203.1	-22.6	-28.2	+15.4	+0.7	0.0	-2.4

* See footnote to table 1.

extent. The final pH values attained in milk and in milk serum are almost the same, but the more heavily buffered milk allowed

more growth, greater fermentation of lactose and probably greater action on the proteins before the limiting pH was reached.

The bromine test for free tryptophane as described in the second paper of this series (Frazier and Rupp, 1928) was found to indicate casein decomposition in milk, but was always negative in milk serum cultures, although lactalbumin, like casein, contains tryptophane.

The determination of amino-nitrogen did not prove useful in the measurement or detection of proteolysis of either casein or lactalbumin in milk or milk serum. With most cultures the results with either the Folin colorimetric or the Sörensen titration method were too close to the limit of error definitely to indicate protein decomposition. Yet a marked decrease in protein nitrogen in the milk serum and a decrease in casein nitrogen in the milk definitely showed proteolysis. Neither is an increase in ammonia an indication of protein decomposition in milk or milk serum, for it has been pointed out that urea-fermenting organisms, like *M. perflavus* or *M. varians* which break down lactalbumin little, if at all, cause more of an increase in ammonia in milk serum than an actively proteolytic organism like *B. vulgatus*. These two micrococci produce a similar increase in ammonia in milk, although they form little ammonia in a casein medium. If they were judged by their ammonia production in milk or milk serum they would be called actively proteolytic when, as a matter of fact, they break down casein or lactalbumin in milk only slightly.

The bromine test for free tryptophane has been found the most satisfactory test for small amounts of casein decomposition. The breaking down of lactalbumin is best detected by analysis for protein nitrogen.

SUMMARY

Ten-day cultures of proteolytic bacteria grown in milk at 30°C. have been quantitatively analyzed before and after incubation for total, casein, non-protein, amino and ammonia nitrogen and ten-day cultures in milk serum have been analyzed before and after incubation for total, protein, non-protein, amino and ammonia nitrogen. Data given are on changes in analytical values.

Most of the organisms which decomposed casein were also able to break down the lactalbumin in milk serum. Some of the cocci did not decompose as great a proportion of the lactalbumin in milk serum as they did of casein in milk. Bacteria like *M. perflavus* and *M. varians* in milk serum caused a considerable increase in ammonia nitrogen which was apparently due to the splitting of urea by these organisms.

For the qualitative detection of caseolysis the bromine test for free tryptophane was satisfactory, but it did not indicate decomposition of lactalbumin.

Neither amino-nitrogen nor ammonia determinations served as a good indication of proteolysis in milk or milk serum, although they proved useful with synthetic media.

Quantitative determinations of casein nitrogen in milk and of protein nitrogen in milk serum served as the best measure of protein decomposition and the best indication of proteolysis.

S. lactis, *L. bulgaricus*, and *L. casei* were apparently able to decompose lactalbumin in milk serum.

Milk serum sterilized by filtration is a good culture medium for many of the common bacteria.

The writers wish to express their thanks to E. F. Deysher of these laboratories for his assistance in the preparation of the milk serum by ultrafiltration.

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STUDIES OF THE ROOT-NODULE ORGANISM OF LUPINUS¹

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Received for publication October 15, 1930

In an earlier paper from this laboratory, Baldwin and Fred (1929a) discussed the characteristics of the root-nodule bacteria and suggested names for five of the common cross-inoculation groups. Specific designation for the organism which forms nodules on the roots of *Lupinus* was not proposed, since the information concerning this cross-inoculation group was so meager.

Schroeter (1886) suggested the name *Phytomyxa lupini* for the organism which formed nodules on *Lupinus luteus* and *Lupinus angustifolius*. Beijerinck (1888) included in a list of seven varieties of *Bacillus radicum*, the organism *Bacillus radicum* var. *Lupini*, which formed nodules on *Lupinus polyphyllus* and *Lupinus luteus*. Later, in 1890, he used the term *Bacillus ornithopi* for the organism forming nodules on serradella. Later results have shown that serradella and lupine cross-inoculate. Dangeard (1926) suggested the species name, *Rhizobium minimum*, for the organism causing nodules on *Lupinus*. His characterization was based largely on the morphological features of the nodule and the organism therein.

In accordance with the rules of nomenclature, it seems that the name *Rhizobium lupini*, Schroeter, *comb. nov.* is the most appropriate for the organism capable of forming nodules on the roots of *Lupinus*. The cultural characters of this organism have not previously been adequately worked out, but most of the

¹ Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

earlier workers have classified it as a relatively scanty and slow grower. In his original description, Beijerinck (1888) did not include the organism from *Lupinus* in the group characterized by "larger, more hyaline colonies." The nodule organisms were placed in two groups by Hiltner and Störmer (1903), and the lupine-nodule bacteria were placed in the group giving slow, or in certain cases no, growth on a gelatin medium. Greig-Smith (1899) observed the extremely slow growth of the lupine organisms on agar plates, and later, in 1906, their failure to form abundant slime on synthetic media, although production was abundant on plant media. More recently Bialosuknia and Klott (1923) reported slow development of the colonies of lupine organisms on agar plates, and equally slow development on agar slants. Also, Müller and Stapp (1925) included lupine organisms in a group characterized by its slow growth. Fred and Davenport (1918) described two lupine cultures as scanty growers, and three as abundant. The rapid-growing lupine cultures were later discarded from the Wisconsin stock cultures as being of doubtful purity.

In 1917, Burrill and Hansen, however, called attention to differences in the rate of growth of the nodule organisms of the different cross-inoculation groups, and described the bacteria from *Lupinus* as fast growers. Four years later, Löhnis and Hansen confirmed the earlier finding with regard to the lupine bacteria. Schönberg (1929) also characterized these organisms by the abundance and rapidity of their growth.

The significance of this disagreement becomes more apparent when the relatively wide difference between the two groups of Löhnis and Hansen is considered.

Many fast-growing cultures have been isolated from *Lupinus* in this laboratory and found capable of forming nodules. Replating of such cultures, however, has never failed to show two types of colony, one fast-growing and one slow-growing. Inoculation of plants with these two types of cultures has resulted in the formation of nodules by the slow-grower, but never by a pure culture of the fast-grower. It may be that two entirely different groups of organisms are capable of forming nodules on *Lupinus*,

but, since this has been recorded in no other case, it is highly improbable.

The present report is a study of the characteristics of eleven cultures of *Rhizobium lupini*. The source of these cultures is given in table 1.

All of the cultures had been repeatedly tested for purity and ability to form nodules. At the end of these experiments the cultures were again tested for purity and nodule-forming ability. Without exception, the organisms were found to be true to name.

Morphology. Mounts of *Rhizobium lupini* taken from a five-day yeast-water mannitol agar slant showed Gram-negative rods

TABLE 1

LABORATORY NUMBER	YEAR SECURED	SOURCE OF CULTURES	
801	1918	<i>Lupinus albus</i>	Sparta, Wisconsin
804	1927	<i>Lupinus nanus</i>	Madison, Wisconsin
805	1927	<i>Lupinus nanus</i>	Madison, Wisconsin
806	1928	<i>Lupinus albus</i>	Madison, Wisconsin
807	1928	<i>Lupinus albus</i>	Hayward, Wisconsin
808	1928	<i>Lupinus albus</i>	Madison, Wisconsin
809	1929	?	Stockholm, Sweden
810	1929	<i>Lupinus mutabilis</i>	Berlin, Germany
811	1929	<i>Lupinus albus</i>	Hancock, Wisconsin
812	1929	<i>Lupinus albus</i>	Hancock, Wisconsin
813	1929	<i>Lupinus albus</i>	Hancock, Wisconsin

varying in size from 1.1 to 3.3 micra in length and 0.1 to 0.3 micron in width. A few coccoid and a few swollen, vacuolated forms were visible.

Cultural characters (yeast-water mannitol agar).² *Rhizobium lupini* produced a scanty or moderate, white, moist, slightly raised, smooth growth on yeast-water mannitol agar. All of the cultures showed an alkaline reaction, agreeing in this character with the other slow-growers. In a medium containing brom thymol blue, or congo red, there was only a slight absorption of the dye. Although variations occurred, none of the cultures showed a rapid

² The media used in this work were prepared according to the formulae given in Fred and Waksman's Laboratory Manual of General Microbiology.

abundant growth such as is exhibited by *Rhizobium leguminosarum*, *Rhizobium trifolii*, *Rhizobium phaseoli*, and *Rhizobium meliloti*. The nature of the growth of *Rhizobium lupini* on this medium is similar to that of *Rhizobium japonicum* and of the organism causing nodules on cowpeas.

Litmus milk. The slightly alkaline reaction, without the production of a serum zone or the reduction of litmus, which was found to characterize all of the *Rhizobium lupini* cultures on this medium, should serve as a purity test for cultures of this organism. Löhnis and Hansen (1921) and Schönberg (1929) working in Löhnis' laboratory, stated that these organisms produced a serum zone on milk. Miss Schönberg suggested, as had Löhnis and Hansen (1921), that organisms isolated from plants of European origin were characterized by the production of a serum zone in milk; those from plants of American-Asiatic origin, by its absence. The inadequacy of this grouping is apparent when it is considered that, as far as is known, the organisms from all the species of one plant genus, with the exception of *Phaseolus*, will cross-inoculate, but in several genera the different species are not all native to the same continent. In the case of the genus *Lupinus*, certain species are apparently natives of Europe and others of America.

Potato and parsnip slopes. Potato slopes were found by Löhnis and Hansen (1921) to serve as an excellent differential medium to separate the root nodule bacteria from the common contaminant, *Bacillus radiobacter*. The meagre, transparent, slimy growth given by the cultures of *Rhizobium lupini* was typical of the root-nodule bacteria. Some of the cultures showed no apparent growth.

On parsnip slopes, prepared in the same manner as potatoes, the organisms gave a white or colorless, viscid, rather scant growth. Growth was very scant or entirely absent, if the parsnips were not washed for several hours.

Glycerol-phosphate solution. Growth of *Rhizobium lupini* on glycerol-phosphate medium varied considerably. Most of the cultures of *Rhizobium lupini* showed a moderate white turbidity, and two strains also formed a surface ring. Four strains produced little or no growth in this medium.

Carrot agar. Carrot agar inoculated with the cultures of *Rhizobium lupini* showed a scant, dry growth. Observation after thirty days disclosed a slight darkening of the agar by the growth of certain of the cultures. Since carrots gave a positive test for tyrosine, it was thought that the color change might be the result of bacterial tyrosinase causing the conversion of tyrosine to the black pigment melanin. The tyrosine content of carrots is variable, and the color phenomenon is not always observed on this medium.

Tyrosine agar. In 1923, Stapp tested several bacterial cultures, including some of *Rhizobia* for the production of tyrosinase, as indicated by the darkening of tyrosine medium. His tests with *Rhizobium lupini* were positive.

Similar tests, using both yeast-water mannitol agar and asparagin agar plus 1.5 gram of tyrosine, were made on these cultures of the lupine organism. A comparison of the growth on the two media showed that it was more profuse on the former with a slight indication of tyrosinase production with all cultures, while on the latter some of the cultures failed to give a positive test. Because of the natural color of the yeast-water substrate, the tyrosinase reaction is not as distinct as on the clear mannitol asparagin substrate.

These results confirmed the supposition that the darkening of carrot agar is due to a tyrosine-tyrosinase reaction. It is also possible that the browning of old cultures of *Rhizobium lupini* on yeast-water mannitol agar may be due to the action of the bacterial tyrosinase on tyrosine, present either in the bacterial cells or in the agar.

Beef-extract peptone gelatin. Growth of *Rhizobium lupini* on beef-extract peptone gelatin was found to be very poor. It was slightly favored by the use of Witte's peptone. Slight liquefaction by four of the strains was noted after three months. The poor growth may have been responsible for the lack of liquefaction in the other cultures. Some of the cultures showed a brownish growth, indicating the possibility of a slight tyrosine reaction. These results are in accord with the results of Hiltner and Störmer (1903) and Fred and Davenport (1918) who found that growth

of the lupine organism was poor on gelatin. Later investigators, Stapp (1924) and Müller and Stapp (1925), observed slow liquefaction by cultures of *Rhizobium lupini* after about a month's incubation.

Dye bacteriostasis. A sucrose peptone solution, plus varying amounts of crystal violet, which Stevens (1925) and Wright (1925) used in differentiating strains of alfalfa and soybean organisms, also brought out variations in these cultures of *Rhizobium lupini*. Certain strains were inhibited in concentrations as low as 1:150,000, while others showed growth at a concentration of 1:15,000.

Fermentation characters. The ability of many of the Rhizobia to utilize as an energy source various carbohydrates has received considerable attention, but little work has been done with *Rhizobium lupini*. Greig-Smith (1911) found that organisms from *Lupinus* produced rather luxuriant slimes from sucrose, glucose, fructose, maltose, and mannitol, but seemed incapable of utilizing lactose. Various investigators [Baldwin and Fred (1927), Walker (1928)] have reported differences in the fermentation characters of the root nodule bacteria. Schönberg (1929) recently made a study of the fermentation characteristics of several of the root-nodule bacteria, including *Rhizobium lupini*.³ Her lupine organisms showed no distinguishing characteristics; however, they tended to resemble the fast-growing group more than the others.

The fermentation characters of the various strains of *Rhizobium lupini* on a nitrate medium plus brom-thymol-blue indicator were studied. To avoid the breakdown of sugars in sterilization, the tubes were placed in the autoclave in small batches, sterilized at 15 pounds pressure for 15 minutes, and rapidly cooled. Careful cleansing of the tubes is important in preventing breakdown of the sugar on sterilization. Definite hydrogen-ion readings were not made, but the reaction was recorded as strongly acid, mediumly acid, or slightly acid, as strongly, mediumly, or slightly alkaline, or as no change in reaction. A representative series of pentoses, hexoses, disaccharides, and trisaccharides was studied. Obser-

³ The medium which was used is stated to be that of Baldwin and Fred (1927); however, the composition was changed to such an extent that the results on the two are not comparable.

TABLE 2
Growth and fermentation characteristics of *Rhizobium lupini* on a nitrate medium containing various pentose and hexose sugars

SUGARS	CULTURES										
	801	804	805	806	807	808	809	810	811	812	813
Rhamnose:											
Growth.....	++	++	++	++	++	++	++	++	++	++	++
Reaction.....	++	++	++	++	++	++	++	++	++	++	++
Arabinose:											
Growth.....	++	++	++	++	++	++	++	++	++	++	++
Reaction.....	++	++	++	++	++	++	++	++	++	++	++
Xylose:											
Growth.....	++	++	++	++	++	++	++	++	++	++	++
Reaction.....	++	++	++	++	++	++	++	++	++	++	++
Fructose:											
Growth.....	++	++	++	++	++	++	++	++	++	++	++
Reaction.....	++	++	++	++	++	++	++	++	++	++	++
Glucose:											
Growth.....	++	++	++	++	++	++	++	++	++	++	++
Reaction.....	++	++	++	++	++	++	++	++	++	++	++
Galactose:											
Growth.....	++	++	++	++	++	++	++	++	++	++	++
Reaction.....	++	++	++	++	++	++	++	++	++	++	++
Mannose:											
Growth.....	++	++	++	++	++	++	++	++	++	++	++
Reaction.....	++	++	++	++	++	++	++	++	++	++	++

Growth: +, scant; ++, scant to moderate; ++++, moderate.

Reaction: ++++, strongly acid; ++, mediumly acid; +, slightly acid; 0, no change in reaction; -, slightly alkaline; ---, mediumly alkaline; ----, strongly alkaline.

TABLE 3
Growth and fermentation characteristics of *Rhizobium lupini* on a nitrate medium containing various disaccharide and trisaccharide sugars

SUGARS	CULTURES										
	801	804	805	806	807	808	809	810	811	812	813
Sucrose:											
Growth.....	++	++	++	++	++	++	++	++	++	++	++
Reaction.....	--	--	--	--	--	--	--	--	--	--	--
Lactose:											
Growth.....	++	++	+	+	+	+	+	+	++	++	++
Reaction.....	--	--	--	--	--	--	--	--	--	--	--
Maltose:											
Growth.....	++	++	++	++	++	++	++	++	++	++	++
Reaction.....	--	--	--	--	--	--	--	--	--	--	--
Trehalose:											
Growth.....	++	++	++	++	++	++	++	++	++	++	++
Reaction.....	--	--	--	--	--	--	--	--	--	--	--
Raffinose:											
Growth.....	++	++	++	++	++	++	++	++	++	++	++
Reaction.....	--	--	--	--	--	--	--	--	--	--	--
Melezitose:											
Growth.....	++	++	++	++	++	++	++	++	++	++	++
Reaction.....	--	--	--	--	--	--	--	--	--	--	--

Growth: +, scant; ++, scant to moderate; +++ moderate.

Reaction: +++ strongly acid; ++, mediumly acid; +, slightly acid; 0, no change in reaction; -, slightly alkaline; --, mediumly alkaline; ---, strongly alkaline.

vations were made at various intervals, but to save space only the two-week readings are reported. In many cases, observations at the end of three or four weeks differed with respect to reaction changes from those reported, apparently depending on the ratio of acid formation to acid breakdown, and the rate of withdrawal of the nitrate or phosphate radicals.

The results of these tests are given in tables 2 and 3. Variations were observed among the cultures, in their growth on, and changes in the reaction of, the various media. *Rhizobium lupini* differed from the fast-growing group of Rhizobia in the fermentation reactions exhibited on several media. Galactose, however, served best, because of the alkaline reaction produced by *Rhizobium lupini*, to differentiate it from all of the fast-growing groups (*Rhizobium phaseoli*, *Rhizobium meliloti*, *Rhizobium trifolii*, and *Rhizobium leguminosarum*). Resembling the slow-growing *Rhizobium japonicum* and the Rhizobium from cowpeas in so many respects, *Rhizobium lupini* could, however, be separated from them by the fermentation reaction on rhamnose and xylose; the initial alkaline reaction is followed much more rapidly with an acid reaction with *Rhizobium lupini* than with the others.

Of considerable interest is the lack of correlation which exists between the fermentative character and the growth of an organism on any one sugar, especially in the disaccharide and trisaccharide groups. Fred (1912) found that growth on sugar media could be very vigorous with less than a 4 to 5 per cent destruction of the total sugar present. A comparison of the growth and reaction changes in the various sugar media with *Rhizobium lupini* is shown in tables 2 and 3. The fermentation of the sugar in the medium with the production of acid is no indication of the extent to which the organism has grown.

PLANT INOCULATION

All of the cultures of *Rhizobium lupini* were tested for nodule production and effect upon the host plant several times during the course of the laboratory experiments. The tests were made on *Lupinus albus*, *Lupinus angustifolius*, *Lupinus hirsutus* and *Lupinus luteus*.

Bacteria-free seeds were planted in sterilized white quartz sand of neutral reaction, which was almost nitrogen-free. The seed was inoculated, after planting and before covering, with a water suspension of the organisms washed from an agar slant. Uninoculated controls were distributed among the inoculated pots. They were watered with sterile water when necessary. Somner's (1928) nutrient solution, minus the potassium nitrate and the silicon salt, was used one or twice a week after the first two weeks of growth.

A representative experiment with *Lupinus albus*, the most frequently used species, is presented in table 4. Variations in plant growth, dry weights, total nitrogen, and to a certain extent in nodulation, were marked. In general, the plants with a few large

TABLE 4
Effect of various cultures of Rhizobium lupini on the growth of Lupinus albus
Eight weeks of age

	Control	801	804	805	806	807	808	809	810	811	812	813
Appearance of plants...	Poor	Very good	Very good	Very good	Good	Fair	Good	Very good	Very good	Very good	Good	Fair
Grams dry weight per plant.....	0.395	0.733	0.720	0.840	0.6	0.613	0.690	0.786	0.726	0.820	0.619	0.478
Percentage of nitrogen.	1.79	2.93	3.17	3.04	3.18	2.06	3.17	3.07	3.36	2.93	3.19	3.21
Number of nodules per plant.....	0	13	13	8	11	20	17	12	12	8	6	12

nodules concentrated on the tap root were the best; but the variation in nodulation was not definitely proportional to the variation in plant development. It is interesting to note that a high nitrogen percentage does not always indicate thrifty plant growth. Plants inoculated with strain 813 gave a very low dry weight, but contained a high percentage of nitrogen. Similar strain variations in respect to the benefit derived from the host plant have been carefully studied by Stevens (1925) with *Rhizobium meliloti*, by Wright (1925) with *Rhizobium japonicum*, by Helz, Baldwin and Fred (1927) with *Rhizobium leguminosarum*, and by Baldwin and Fred (1929b) with *Rhizobium trifolii*.

Variations in results also occurred on different species of lupinus. The same culture of bacteria caused the formation of round,

medium-sized nodules on the top of the lateral roots of *Lupinus albus*, while on *Lupinus angustifolius* and *Lupinus luteus*, the nodules were extremely large and usually formed a collar around the tap root near the surface of the soil. The nodules on *Lupinus hirsutus* were small and either on the tap root or the lateral roots. As noted by Kirchner (1895), *Lupinus hirsutus*, even when planted in the same inoculated pot with other species which showed nodules, often failed to show any nodulation. It was observed that cultures which gave excellent results on *Lupinus albus* often gave only fair results on other species of lupine, or *vice versa*. Hiltner, as early as 1902, noted that there is a marked difference in the behavior of the same organism on blue and yellow lupines.

SUMMARY

In accordance with the rules of nomenclature, it is proposed that the name *Rhizobium lupini* (Schroeter) *comb. nov.* be given to the organism causing the formation of nodules on *Lupinus* sp.

Rhizobium lupini is a Gram-negative organism, producing a scant to moderate growth and alkaline reaction on yeast-water mannitol agar, a slightly alkaline reaction without reduction and without the formation of a serum zone in litmus milk, a meagre growth on potato and parsnip slopes and on carrot agar. Slight production of tyrosinase by all the strains was noted on tyrosine media. Growth on beef-extract peptone gelatin, and liquefaction of this medium, sensitivity to crystal violet, and the nature of nodulation and the benefit to the host plant varied among the different strains; strain variations also occurred in fermentation characters, but the results with certain sugars were definite enough to serve as a means of separating *Rhizobium lupini* from the other root-nodule bacteria.

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FATE OF FRESH WATER BACTERIA IN THE SEA

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Received for publication October 30, 1930

Fresh water bacteria¹ are continually carried into the sea by streams, leachings from the shore, dust and migrating animals. Salt water bacteria are also carried into fresh water. The interchange of bacteria between fresh water and the sea is so extensive and continuous that unless the environmental change is inhibitory we may expect to find many species common to both environments.

The fate of fresh water bacteria in the sea is of considerable importance. The classification of marine bacteria would be greatly simplified if it were established that fresh water bacteria do not maintain themselves in the sea. If we must consider that any bacteria taken from the sea may be identical with fresh water forms the identification becomes more difficult. The higher forms of animals and plants are mainly distinct. To what degree this is true of the simpler forms, particularly bacteria, remains to be determined.

The fate of the fresh water bacteria in the sea is of interest in connection with the general biology of that region. If the fresh water bacteria survive and carry on their activities as in fresh water we may apply much that we know of fresh water bacteriology to an analysis of changes occurring in the sea. The renewal of the food supply of the ocean as of the land depends, to a large extent, on bacterial decomposition. The fresh water and soil bacteria are responsible for the formation of the nitrates and other decomposition products favorable to plant life washed into the sea from the land. Do they also take part in the decom-

¹By the term fresh water bacteria is meant all bacteria carried into the sea from the land.

position occurring in the sea? The sea differs from the land in that it could support life in so far as the food supply is concerned without decomposition processes because of additions from the land. Decomposition occurs in the sea, however, and is so vitally important to the fertility of that region that an exact knowledge of the organisms involved and the stage in the process for which each is responsible is important.

The main change in the environment to which fresh water bacteria are exposed upon entering the sea is an increase in the concentration of salts, mainly sodium chloride. There are other changes associated with depth but these apply only to parts of the sea. It appears to us that if fresh water bacteria can survive exposure to the salt concentration of the sea they should survive and carry on their activities when carried into the sea to the same extent as when carried into large bodies of fresh water such as lakes.

A number of factors such as organic matter, temperature, light, biological antagonism, number of organisms, age, hydrogen ion concentration, and amount of oxygen affect bacterial life in the sea but these are common to fresh water and probably are not determining factors in the survival of fresh water forms in the sea. For an analysis of factors affecting survival in water and saline suspension the reader is referred to Ballantyne (1930).

The experiments described in this paper deal with (1) the survival of river bacteria in sea water, (2) their ability to carry on their activities, (3) their ability to adapt themselves to concentrations of salt greater than those present in the sea.

EXPERIMENTAL

Materials

Standard beef extract broth and agar with a pH of 7.1 to 7.3 unless otherwise stated, were used for culture and plating work. Six per cent glycerol was added to the agar slants. The sea water was obtained in the middle of the Strait of San Juan de Fuca at full tide and sterilized by heat. Twenty-one organisms were isolated from the Palouse River. These consisted of cocci, bacilli, actinomyces, including aerobes and facultative anaerobes. Not all were used in each experiment.

Experiment 1. To compare the survival of fresh water bacteria in sea water and fresh water

Twenty-four-hour broth cultures of 9 river organisms were prepared and inoculated into sea water, pH 7.7; tap water, pH 7.5; and broth, pH 7.5. After shaking, 1 cc. amounts were plated out to serve as a standard for determining the death curve. The tubes were incubated at 20° to 22°C., for seventy days. A similar set of tubes was prepared and incubated at 7° to 12°C. At intervals of from one to five days, 1 cc. amounts were plated out and compared with the standard.

In the cultures held at 20° to 22°C., an immediate and fairly comparable decline in numbers occurred in the sea and tap water with an increase in the number of organisms in the broth. All the cultures in both the sea water and tap water remained viable for forty-five days. In general the decline in numbers was somewhat greater in sea water. Between the forty-fifth and seventieth day, 3 of the cultures in sea water died out. All the cultures in tap water survived. In the broth the number increased up to twenty to twenty-five days and then remained constant or decreased. All were viable at seventy days.

In the cultures held at 7° to 12°C., the death rate was more rapid. Four cultures in sea water and 3 in tap water died out by the fortieth day. Three cultures in sea water and 3 in tap water survived seventy days, when the experiment was discontinued. In the broth the drop in numbers began earlier, about the fifteenth day, and two of the cultures failed to survive the seventy days.

The results obtained indicate that fresh water bacteria survive in the sea for a considerable time. In general, fresh water bacteria do not survive as long in sea water as in the tap water used. They survive longer at 20° to 22°C., than at 7° to 12°C. The results also indicate that some of the species at least will survive longer in sea water at 20° to 22°C., than in tap water at 7° to 12°C.

The results obtained with the broth suggest that the organism survives as long and possibly longer in the presence of organic matter. Ballantyne and Winslow and associates have shown that organic matter is a factor in survival. Undoubtedly there was

more organic matter in the sea water than in the tap water. The tap water was from a deep well and practically free from organic matter. As a possible off-set to the effect of such organic matter as may have been present in the sea water the pH was 7.7 as compared with pH 7.5 of the tap water. An experiment made to determine the effect of pH on the toxicity of salt demonstrated an increase in toxicity with an increase in pH. This is slight within a range of pH 0.2 and apparently less in the presence of organic matter.

For bacteria to multiply and survive indefinitely in either fresh or sea water organic matter must be present. Experiment 1 demonstrated the ability of fresh water bacteria to survive for a considerable time in sea water in the presence of such organic matter as was there. Apparently no multiplication took place. It seemed advisable to determine whether the organisms would multiply and carry on their activities as in fresh water. Their ability to do so would indicate that the very young cells can resist sea water. In general, bacteria have a better chance to survive exposure to bactericidal and bacteriostatic agents in the presence of organic matter.

Experiment 2. To determine whether fresh water bacteria can decompose organic matter in the sea

Meat tubes were prepared by adding canned salmon to sea water. Two series of such tubes were inoculated from forty-eight-hour cultures of 20 river organisms. One series of tubes was incubated at 7° to 12°C., and the second series at 37°C. The tubes were examined at ten and twenty days.

Of the 20 cultures held at 7° to 12°C., eleven showed no growth. In the remaining nine cultures there was growth and decomposition to some extent, varying with the organism. Growth occurred in all the tubes incubated at 37°C. The amount of growth and decomposition of the salmon varied with the organism. In 4 tubes the growth was slight. Three of the organisms grew nearly as well at 7° to 12°C., as at 37°C. The optimum temperature for growth for some of the organisms may have been between those used.

The results obtained indicate that many fresh water bacteria can grow in sea water in the presence of organic matter. It follows that the young cells can survive exposure to salt water under the conditions of the experiment.

Since fresh water bacteria were found to survive and multiply in sea water it was decided to determine how long they would survive in presence of organic matter in salt concentrations greater than those present in sea water. Results obtained under such conditions should not be compared with results obtained with sea water due to other factors present, but they do indicate the effect of sodium chloride on the organisms and this salt appears to be the dominant factor affecting the survival of fresh water and land organisms in the sea.

Experiment 3. To determine the tolerance of fresh water bacteria for sodium chloride concentrations greater than those found in the sea

To plain broth, pH 8.0 was added sufficient NaCl to make molar concentrations of 0.3, 0.48, 0.7, 0.9, 1.5, 2.0. The sea has a salt concentration varying around 0.48 M. Each series of tubes was inoculated with a twenty-four-hour culture of 1 of 9 river organisms. Plain broth was used as control. The tubes were incubated at 7° to 12°C. Transfers at three-day intervals were made to determine viability. The experiment terminated at seventy days.

Of the 9 organisms 3 were spore-bearers. All of these survived exposure to salt concentrations greater than that found in the sea but none survived the seventy days in a 2.0 M concentration. All three survived fifty days in all concentrations. The length of survival of each varied with the salt concentration.

Of the 6 non-spore-bearing organisms one survived seventy days in concentration equal to or slightly greater than that found in the sea. This one did not survive as long as the spore-bearers in the greater concentrations of salt. The other 5 non-spore-bearing organisms in salt concentrations of 0.7 molar survived from sixteen to forty-four days, in 2.0 molar concentrations two to twenty-five days. It is evident that bacteria in the presence

of organic matter will survive for a considerable time in salt concentrations greater than that found in the sea. However, salt in concentration of 0.3 M, which is less than that of the sea, reduces viability. Comparative survival of the organism in salt water, salt broth, and sea water with equal concentrations of NaCl was not determined. Undoubtedly organic matter is a factor in survival. The work of Ballantyne and others supports these views. Our experiment was not conducted long enough to determine to what degree organic matter contributed to survival in the presence of salt.

We have assumed that in so far as the fate of fresh water bacteria in the sea is concerned the main factor is the sodium chloride concentration. Other factors effect bacterial life but they are common to both sea and fresh water. Other salts are present in sea water but they are not in sufficient concentration in themselves to inhibit fresh water bacteria. The possibility of their increasing or decreasing the toxic action of salt was considered. An experiment was made to determine this point by adding the salts of KCl, MgSO_4 , CaCl_2 , and K_2SO_4 , to a salt solution in concentrations approximating those reported for sea water. The toxic action of the sodium chloride was not increased and apparently in one case was decreased. The combined action of salts on bacteria has been critically investigated by Winslow and Dolloff (1928). Apparently salts other than sodium chloride are not determining factors in the survival of fresh water bacteria in the sea. Gases probably do not affect bacterial survival to any greater extent in the sea than in fresh water.

The preceding experiments deal mainly with the new factors encountered which might affect bacterial survival in the sea. The following experiments deal with certain factors affecting bacterial life in any environment but which have significance in the interpretation of experimental results on the effect of salts on survival.

Bacteria of all ages and conditions of dormancy enter the sea. In previous experiments we used twenty-four-hour cultures. The following experiment was made to determine the effect of age on survival. Experiment 2 demonstrated that young cells can survive in the presence of organic matter, but did not indicate their

comparative resistance. All cells must be young before they become old. If the young cells are less resistant this may lead to a disappearance of the species in the sea.

Experiment 4. To determine the comparative resistance to salt of young and old bacterial cells

Tubes of plain broth containing a 4 molar concentration of NaCl were inoculated with 14 river organisms. A series was prepared for each species from cultures aged 4, 12, 24, 48, 72, 120, 168, and 192 hours. Incubation was at room temperature. Transfers were made at one-half hour intervals to determine the death time.

The results obtained indicate that the young cells are much more sensitive to the action of salt than older cells. The cells reached their greatest resistance at twenty-four to forty-eight hours. Cultures older than forty-eight hours either retained their tolerance for salt or showed a slight reduction up to one hundred ninety-two hours.

The bacteriostatic and bactericidal action of most if not all substances is in general increased with an increase in temperature. An experiment was made to determine the effect of heat on the bactericidal action of a strong sodium chloride solution. A 4-molar concentration at 7° to 12°C., room temperature and 45°C. was used. The organisms survived longer at the lower temperatures. In weaker salt concentrations, i.e., in ordinary sea water with a molar concentration of about 0.485 the organisms survived longer at 20° to 22°C. than at 7° to 12°C. These results are in accordance with what we know of the effect of heat on antiseptics and the survival of organisms in cultures. They suggest that the chance of fresh water bacteria to survive in the sea may vary somewhat with the temperature.

The fate of an organism in a new and unfavorable environment depends to some extent upon adaptation. All cells have the power of adaptation. It has been demonstrated that bacteria can adapt themselves to both physical and chemical factors affecting life. Burke, Ulrich and Hendrie (1928) demonstrated that *Staphylococcus albus* can develop increased tolerance for acrifla-

vine, one of the antiseptic dyes, within six hours. Since the salt in the sea is in sufficient concentration to affect the survival of fresh water bacteria the ability of the organism to develop increased tolerance for salt becomes a factor affecting their existence.

Experiment 5. Bacterial adaptation to sodium chloride

Saline broth was prepared with molar concentrations of 0.45, 0.5, 0.55, 0.6, 0.65, and 0.7 of sodium chloride. Tubes with a 0.45 molar concentration were inoculated with 20 river bacteria. As controls, cultures in plain broth were made each forty-eight hours. After forty-eight hours incubation at 37°C., transplants were made to broth containing a 0.5 molar concentration. This procedure was repeated until the broth containing the 0.7 molar concentration of salt was used. Fourteen of the organisms survived this treatment. As a means of detecting increased tolerance for salt, transfer of the 14 organisms and their controls of the same age were made into broth containing a 4 molar concentration of salt. This was kept at room temperature. At the beginning and at thirty-minute intervals up to seven hours transplants to agar slants were made. The results were read after twenty-four hours incubation at 37°C., and twenty-four hours at room temperature.

Two of the organisms showed decreased tolerance for salt, 2 had the same tolerance as the controls and 10 showed increased tolerance, some living more than twice as long as the controls. This experiment demonstrated that a large percentage of fresh water bacteria can develop increased resistance to salt, starting in a concentration in excess of that frequently encountered in brackish water about the mouths of streams.

It has been demonstrated in other experiments in this laboratory that injurious effects of exposure to an unfavorable environment may be transmitted. An organism after exposure may be less rather than more resistant. This depends on the concentration. In demonstrating increased tolerance or adaptation to any agent it is advisable not to expose to too great a concentration. We are of the opinion that the reason the 4 organisms did not adapt themselves is that conditions were not favorable. This

applied also to the six strains that died out. The following experiment favors this view.

In the former experiment we utilized the process of exposure and transplants to greater concentrations of salt in broth. In the following experiment we used exposure to salt in agar as a means of selecting the most resistant organisms each time and using them for subsequent exposure. This represents a process of exposure and rigorous selection to alter the cultures.

Salt agar plates were prepared by adding sodium chloride in molar concentrations of 0.8, 0.85, 0.9, 1.0, 1.5, and 2.0. The 21 river organisms were streaked on these plates. The plates were incubated twenty-four hours at 37°C. and twenty-four hours at room temperature. From colonies on the greatest concentration of salt agar streaks were made on agar containing greater salt concentration. This was done four times. All the 21 organisms at first grew on salt agar containing 0.85 molar concentration. The majority grew on a 1.0-molar concentration in agar, a concentration about twice that occurring in the sea.

As the result of 4 transfers on salt agar all the organisms including those that failed to acquire tolerance in the preceding experiment in which salt broth was used, acquired greater tolerance for salt. All the organisms except 1 developed resistance to salt twice that tolerated at first and by the control, or from 4 to 5 times that occurring in the sea. It should be mentioned that a bacteriostatic agent is not as effective in agar as in broth or water.

DISCUSSION

The fate of fresh water bacteria in the sea, i.e., their ability to maintain themselves, will depend on how long they can survive in the absence of organic matter, whether they can carry on their activities and multiply in the presence of organic matter and to what degree they can adapt themselves to the new factors affecting their survival. Our experiments suggest that many fresh water species of bacteria can under favorable conditions or in favorable regions maintain themselves in the sea.

In our experiments we have assumed that the main distinctive

factor affecting the survival and growth of fresh water bacteria in the sea is the concentration of sodium chloride and that if this salt does not destroy these organisms they will be able to maintain themselves in the sea to the same extent as in large bodies of fresh water.

Other factors affecting bacterial life in general are common to the sea and large lakes. They vary in different areas of the sea as in fresh water. We can exclude gases as interfering in the sea to any greater extent than in fresh water. These vary with depth, animal and plant life and sunshine. Salts other than sodium chloride are present in small concentration and are probably not a determining factor in the sea. Our experiments demonstrated that they do not increase the toxic action of sodium chloride. In the open sea the water is slightly on the alkaline side of neutrality, about pH 8. In tide pools it becomes more alkaline. In the great depths it is less alkaline but never neutral. It is possible that these factors vary sufficiently so that some areas are less favorable than others to the survival of fresh water bacteria.

Bacteria entering the sea at the mouths of large rivers are exposed to brackish water for some time. Adaptation to increased salt concentration may be established before exposure to pure sea water occurs. This is possibly a factor affecting survival.

Certain other evidence has a bearing on the fate of fresh water bacteria in the sea. It is well known that intestinal organisms survive exposure to brackish water. Typhoid and other diseases are spread by sewage contaminating oyster beds. Bacteriological examination of fish canneries and their products suggests survival of fresh water bacteria near shore. For purposes of isolation and identification bacteria are sometimes exposed to or grown in salt concentrations greater than those present in the sea. Hill and White (1929) have shown that cocci may be isolated in agar containing 6 to 15 per cent salt. Their work shows that many bacteria can be grown in media containing salt greatly in excess of that found in the sea.

Korineck (1926) found that bacteria from various sources other than the sea grow on media containing sea water. He believes however that in the sea they probably exist in a dormant state and do not take part in the decomposition of organic matter.

The death rate of fresh water bacteria in the sea is undoubtedly high. It is high in large bodies of fresh water where the water is so greatly in excess of organic matter that there is no multiplication. Sunshine at the surface, sedimentation, and antagonisms account for the destruction of great numbers. Bacterial populations vary as these factors vary. We believe, however, that many fresh water bacteria carried into the sea can maintain themselves and carry on their activities as in fresh water and contribute much to the biology of the sea. This possibility must be given consideration in the solution of some of the remaining unsolved problems connected with marine biology. It appears probable that the great fertility of the tide area is due in part to the fresh water bacteria surviving there. Even though they do not survive in the ocean they must still be considered as dominant factors in the formation of the nitrates washed into the sea from the land. These nitrates as well as other decomposition products resulting from bacterial activity are factors in the maintenance of a rich littoral flora and fauna. Furthermore, the bacterial cells serve as a food supply for certain protozoa.

The fate of marine bacteria reaching fresh water remains to be determined and is under investigation. The work of Korineck (1926) and of Lipman (1926) suggests that at least some of the marine bacteria have little chance to survive in fresh water.

CONCLUSIONS

1. Fresh water bacteria survive in sea water nearly as long as in tap water. They survive longer at 20° to 22°C., than at 7° to 12°C. Some survive longer in sea water at 20° to 22°C., than in tap water at 7° to 12°C. They probably survive longer in the presence of organic matter.
2. Some fresh water bacteria survive for a considerable time in broth containing salt in concentration 2 to 4 times that found in the sea.
3. Many fresh water bacteria can develop increased tolerance for salt twice that shown originally and grow in concentration in excess of that found in the sea.
4. Salts in the sea other than sodium chloride do not reduce viability of fresh water bacteria.

5. Cells of young cultures are more susceptible to salt than the cells of older cultures. The greatest resistance is reached in twenty-four-to forty-eight-hour cultures.

6. Fresh water bacteria can decompose organic matter in the sea.

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THE EFFECT OF STERILIZATION OF MEDIA UPON THEIR GROWTH PROMOTING PROPERTIES TOWARD BACTERIA

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Received for publication November 3, 1930

Data presented by Fulmer and Huesselmann (1927) showed that sterilization of yeast growth media under pressure produces a yeast growth stimulant. The production of the stimulant was always associated with caramelization of the medium. The standard medium contained per 100 cc. 0.188 gram ammonium chloride, 0.180 gram dipotassium phosphate and 10 grams of sucrose. Sterilization under pressure of the sucrose and ammonium salt (preparation A) and of sucrose and dipotassium phosphate (preparation B) likewise led to the formation of the stimulant. For each preparation there was an optimum concentration of the caramelized medium for growth, the exceeding of which depressed the growth. The preparations differed in stability toward hydrochloric acid; the ammonium chloride preparation was not decolorized by addition of the acid, i.e., is "acid fast" while the phosphate caramel was practically decolorized by such a treatment. The various preparations were not significantly different in degree of stimulation. The stimulation was shown to be due to changes produced in the sugar by the reagents and was not a pH effect.

Data presented recently by Lewis (1930) showed that "media containing glucose, maltose, lactose, galactose or levulose with various nitrogenous compounds when sterilized at 122°C. for fifteen minutes" formed substances which inhibited the growth of *Phytomonas malvaceara*. He attributes the inhibitory effect to "conversion of the nitrogen compound into a form which is not suitable for assimilation by some species of bacteria." He

did record experiments with a medium not containing nitrogen. In the treated medium containing sodium ammonim phosphate, the growth of the following organisms was not inhibited, *Serratia marcescens*, *Salmonella enteritidis*, *Escherichia coli*, *Aerobacter aerogenes*, *Pseudomonas fluorescens* and *Ps. aeruginosa*, while the growth of *Phytomonas melvaceara* was inhibited. With peptone as a source of nitrogen, the treated medium inhibited the growth of *Staphylococcus albus*, *Staph. aureus*, *Sarcina lutea*, *Bacillus mycoides*, and *B. anthracis*, while the non-inhibited species included *E. coli*, *B. subtilis*, *A. aerogenes*, *Serratia marcescens*, and *Ps. fluorescens*.

During the course of work in these laboratories on the fermentation changes produced by *Aerobacter pectinovorum* it was noted that growth was always better in the medium sterilized for fifteen minutes at 15 pounds pressure than in the medium sterilized by filtration. The medium contained per 100 cc., 0.6 gram ammonium chloride, 0.2 gram dipotassium phosphate and 5 grams of glucose. These results were analogous to those reviewed above for yeast and indicated the production of a bacterial growth stimulant during the process of sterilization.

In order to determine the relative degree of stimulation brought about by caramelization, the flasks of media in each experiment were adjusted to the same pH and inoculated with one drop of an actively growing culture. The flasks were watched closely for the first appearance of growth and the time recorded.

To four of eight flasks containing 5 per cent glucose solution, ammonium chloride and dibasic potassium phosphate were added in the right amounts. The medium was then sterilized at 15 pounds pressure for fifteen minutes and the salts added to the other four flasks of medium. The pH was adjusted to 6.8 in all the flasks and they were inoculated. Within twenty-four hours the four flasks containing the caramelized medium showed growth, while it was seventy-two hours before growth was apparent in the four which were not caramelized. This would indicate that either the caramel or some substance which is formed simultaneously with the former acts as a stimulant toward the organism used.

To determine if this substance might act as a stimulant under different conditions, two other similar series were arranged, one at a pH of 7.05 and the other at 6.6. Within twenty-four hours after inoculation all media which contained some caramel, showed growth. The uncaramelized media showed growth after seventy-two hours; apparently, stimulation may occur at any pH suitable for the growth of the organism.

In order to determine whether or not an increase in the concentration of caramel would cause an increase in stimulation, the medium containing phosphate and ammonium chloride was sterilized under 15 pounds pressure for five hours which caused an

TABLE 1

FLASK NUMBER	PER CENT OF CARAMELIZED SOLUTION	PER CENT OF UNCAMELIZED SOLUTION
1	0	100
2	0	100
3	0	100
4	10	90
5	20	80
6	30	70
7	40	60
8	50	50
9	60	40
10	70	30
11	80	20
12	90	10
13	100	0

intense but clear coloring of the medium. This corresponds to the standard caramel solution prepared by Fulmer and Huesselmann, and portions of it were added to uncaramelized medium to form the concentrations shown in table 1.

The flasks were then sterilized in live steam at atmospheric pressure for ten minutes. The pH was adjusted to 6.8 and the flasks were inoculated. Flasks number 1, 2, and 3 were used as checks. Twelve hours later there was growth in all flasks which contained caramel. It was very evident that the media with the higher per cent of caramel contained the most growth. In fact the medium consisting of 100 per cent caramel solution

appeared to have the greatest stimulating effect. The medium which contained no caramel required about fifty hours for appearance of growth.

A commercial caramel of unknown origin and composition was tried. A slight stimulation was observed but not comparable to that obtained above.

Another caramel was prepared as follows: 100 grams of glucose were heated to a temperature of 200°C. and held at this temperature for twenty minutes, cooled and dissolved in water to make 250 cc. of solution. Varying quantities of this solution were added to an uncaramelized medium. This caused a slight stimulation but not comparable to that obtained by sterilization of the medium.

TABLE 2

ORGANISM	TIME FOR GROWTH TO BE APPARENT	
	Caramelized medium	Uncaramelized medium
	hours	hours
<i>Aerobacter faeni</i>	12	36
<i>Esch. freundii</i>	15	36
<i>Actinomyces</i> , sp?	22	
<i>Aerobacter aerogenes</i>	12	24
<i>Serratia marcescens</i>	15	15*
<i>Esch. coli</i>	15	15*
<i>B. subtilis</i>	36	36*

* The growth was greater in the caramelized medium.

An attempt was made to determine whether or not the stimulant produced during sterilization could be removed by decolorizing the medium with charcoal. A commercial charcoal, Norite A, was boiled and washed several times with distilled water to remove any soluble substances which might be present. A little of this charcoal was added to each of four flasks, two of which contained caramelized media and two, uncaramelized. After standing for three to four hours the charcoal was filtered off and the medium sterilized in live steam at atmospheric pressure for ten minutes. The pH was adjusted to 6.8 and the flasks were inoculated. Within twelve hours growth was very evident in

all four flasks. The experiment was repeated with the same results. It was then noticed that traces of charcoal had passed through the filter and were present in the medium. The experiment was again repeated and in this case the medium was filtered a second time through a Berkfeld type of filter. Within fifteen hours there was a heavy growth in the medium which had contained the caramel. No growth was evident in the other flasks at the end of ninety-two hours. This proves without doubt that the stimulation is not due to the caramelization of the medium but to some substance which is produced at the same time as is the caramel. The stimulation in this case cannot be attributed to the effect of the charcoal, since each flask had precisely the same treatment.

It seemed desirable to try the effect of a caramelized medium upon other organisms capable of growth on ammonium salts as a source of nitrogen. The results are given in table 2.

The last four organisms in table 2 are four of the five organisms which Lewis was able to grow on the caramelized medium. The data above show that these organisms are actually stimulated. It may be concluded, then, that the sterilization of media under pressure may lead to the production of growth stimulants for yeast and for the bacteria studied. Decolorization of the caramelized medium with charcoal (Norite A) does not remove the bacterial growth stimulant.

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CONJUNCTION OF DISEXUAL FORMS IN ASCOBACILLUS

A PRELIMINARY REPORT

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Received for publication, November 6, 1930

For some months we have been studying what we believe to be an instance of disexual conjunction occurring in an apparently hitherto undescribed species of *Ascobacillus*, obtained from a piece of burlap. This phenomenon was observed in the course of a series of studies of the effect which long continued variations of temperature and hydrogen-ion concentration may have on the morphology of organisms. The continuance of this conjunction phase throughout a period of seven months seems worthy of a preliminary report, though other noteworthy results and conclusions are not yet ready to publish.

There are many references in the literature to evidence of some form of conjugation in bacteria; but it is beyond the scope of a preliminary report as brief as this one to attempt anything like a complete review of the subject. Löhnis, in his exhaustive review of the literature up to 1918, deals at length with the question of sexual phases of bacterial life cycles. We have not yet secured a copy of Enderlein's recent work, but he is said to report an elaborate life cycle with clearly recognizable disexual forms, such as have been reported for the cholera vibrio by earlier workers (Löhnis, p. 202). Among the others who have devoted much study to this subject, Mellon and Almquist have contributed richly to our knowledge of this bacterial phase.

Our organism is a motile rod, the typical bacillary forms measuring from 1μ to 2.5μ by 0.3μ to 0.6μ (fig. 1). It possesses peritrichous flagella, and does not retain Gram's stain. It grows

easily on all laboratory media, producing acid but no gas in glucose, lactose, and sucrose, acidifying and coagulating milk after three weeks, and slowly liquefying gelatin. It develops a tough pellicle in broth, with turbidity throughout the media, a new pellicle repeatedly reforming when the culture is disturbed while the old pellicle breaks and falls to the bottom of the tube. On agar slants the growth is confluent, moist, and translucent yellow. On agar plates the colonies are moist, slightly convex, and cream-white, later becoming yellow.

The cultures of this *Ascobacillus* have a strong and very unpleasant odor, slightly resembling that of *Esch. coli*. The genus characteristic of this organism is shown in its formation of "families," smoothly contoured oval masses of organisms that occur in clusters of from two to many such ovals embedded in a hyaline matrix, which resembles a large gelatinous capsule (figs. 1 and 2). These masses later break up into numbers of rods. The name *Ascobacillus heterozygeus*¹ is proposed for this new species.

This organism was obtained in pure culture by repeated plating and fishing before transferring it into a series of broth media in connection with the various studies already mentioned. It happened that two cultures made in meat extract broth of pH 4 developed no pellicle and were not examined with the other cultures of the same date, though their turbid appearance indicated abundant growth. Not until after a lapse of two months were these tubes examined by stained smears, and then organisms of strikingly bizarre appearance, strongly indicating conjugation, were found. Hanging drop mounts were immediately examined and for the past seven months many such preparations have been studied for periods of several hours at a time. In these pH 4 broth preparations the following phenomena have been observed.

Large swollen crescent or spindle-shaped motile organisms were heavily interspersed among very active short rods, similar to the heavily staining rods of frequent occurrence in all other cultures of this organism which had been studied. Darting about

¹ Moriyama, Y.: M.S. thesis, 1930, Teachers College, Columbia University. (Filed, but not published.)

the larger spindles, these shorter active rods would strike against them and swim off again, often three or four small organisms hovering about a larger one. Sometimes one of the small bacilli, impinging upon a large spindle at an oblique angle, remained firmly attached to it; and a rapid shortening and curving of the little bacillus occurred, until it assumed the form of an oval attached by a very short stalk to the side of a larger spindle or crescent (figs. 3 and 5₂ to 7). One such pair required but seven minutes for the change from a free bacillus to an attached, thick, curved oval. Of the dozens of paired organisms which have been watched, none has ever been seen to separate again.



FIG. A. DRAWING OF PAIRED ORGANISMS STUDIED IN UNSTAINED HANGING DROP PREPARATION

The crescent measured 6.39μ

The further history of these paired forms is likewise of interest. During the course of hours the appearance of the organisms changes and becomes markedly granular. This change seems first to become evident in the large spindle-shaped cell, and, at least in those organisms which have transformed very slowly, it may be preceded by a spiral organization of the chromatin, such as Mellon has found accompanying conjugation in the colony-typhoid group (figs. 6 and 7). The spiral seems to be connected with, or to extend into, the attached knob (fig. A), and its identity is later lost as both members of the pair become completely granular (fig. 8). The entire substance of both of the cells seems to pass into a granular state. Thus far we have seen nothing to indicate that any outer portion of the cell is discarded as the cells lose their outline and become a mass of somewhat refractile

tiny granules, or symplasm. In broth cultures the final stages of this transformation from clearly distinguishable organisms to symplasm seem to take place in the bottom of the test tube, and material for study taken from this location shows large groups of paired organisms matted together in all stages of granulation.

Microscopically, this symplasm is hard to study, for it washes from the slide so easily, with even such a simple stain as aqueous fuchsin or methylene blue, that little is left of the smear. Cultural methods have been hardly more satisfactory. We transferred with a fine Pasteur pipette two or three drops of this symplasm culture to fresh pH 4 broth tubes. Throughout a period of six weeks the tubes were thoroughly shaken several times. During the first two or three weeks no clouding was visible, but at the end of six weeks symplasm had developed in sufficient quantity to make a distinct granular sediment in the bottom of the tube. This sediment, sufficient to make the broth cloudy when the tube was shaken, soon settles again, leaving the broth clear. Symplasm from this tube, studied in hanging drop mounts sealed with vaseline, has noticeably increased in amount during the course of a week. It has not grown when transferred to agar. From time to time we have thought that we were observing the regeneration of short rods from this amorphous material. Though the granules in this mass of symplasm do not show Brownian movement, occasionally we have found activity at or near the edge of a clump of granules, and we have seen a very short square-ended bacillary form break from a cluster of granules and bob about in the surrounding medium, like a free-swimming organism. It seems, however, to fail to establish itself as a bacillus, apparently returning to the symplasm state.

Studies are in progress to determine the controlling factors in the change from a culture of typical rods to one in which spindle-shaped and pairing organisms prevail, and to observe the intermediate processes. There are indications that the spindles themselves are formed by the earlier fusion of individuals, though this is but infrequently apparent (figs. 4 and 5). Inoculations to agar plates and agar slants from these broth tubes of pairing organisms have yielded no growth at any time. Simultaneous

transfers from these two months old pH 4 broth cultures to fresh pH 4 broth gave, in twenty-four hours, abundant growth of the same bizarre pairing forms. Similar broth transfers made three months later gave only symplasm. Yet after several months, we still find in the original pH 4 tubes, and also in the five months old sub-transfers from them, a few active paired organisms in almost every drop examined. The number of uniting organisms in both sets of tubes is now decreasing, however.

The term conjunction is used for the phenomena here observed, since in some instances, at least, more than two organisms are concerned with this fusing, as in figure 5; and we follow Löhnis in reserving the term conjugation for the union of two organisms. From the accompanying photographs the conclusion might perhaps be drawn that we are dealing with lateral buds, but this is not the case. Careful study of the formation of these oval projections precludes such a possibility. We have seen instances of lateral budding in our *Ascobacillus*, and we are at present observing very active budding in an unusual spore-forming organism, but these phenomena are quite distinct from the ones with which we are here concerned. Conjunction in bacteria is not a newly observed phenomenon, and varying forms of it have been reported from time to time. The conjunction described for *Ascobacillus* is but one of the types which occur in bacteria. The lateral application of similar organisms (fig. 9) has already been reported by us for an organism which we have since tentatively named² *Vibriothrix polymorpha*. So far as we are aware, however, this is the first time that conjunction of dissimilar or disexual forms has been reported in this country. It is hoped that further studies now under way with a longer series of media will yield more complete information regarding these reproductive phases.

² Pease, D.: M.S. thesis, 1930, Teachers College, Columbia University. (Filed, but not published.)

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PLATE 1

FIG. 1. *Ascobacillus heterozygeus*, from a five-day-old pH 9 broth culture, showing typical rods and young "families" of two to eight oval masses. $\times 1425$.

FIG. 2. *Ascobacillus heterozygeus*, from a twenty-five-hour-old pH 9 broth culture, showing young "families" and older oval groups, some of which are in chain formation. $\times 1425$.

FIG. 3. *Ascobacillus heterozygeus*, from three-month-old pH 4 broth culture, showing paired organisms and small masses of symplasm. $\times 760$.

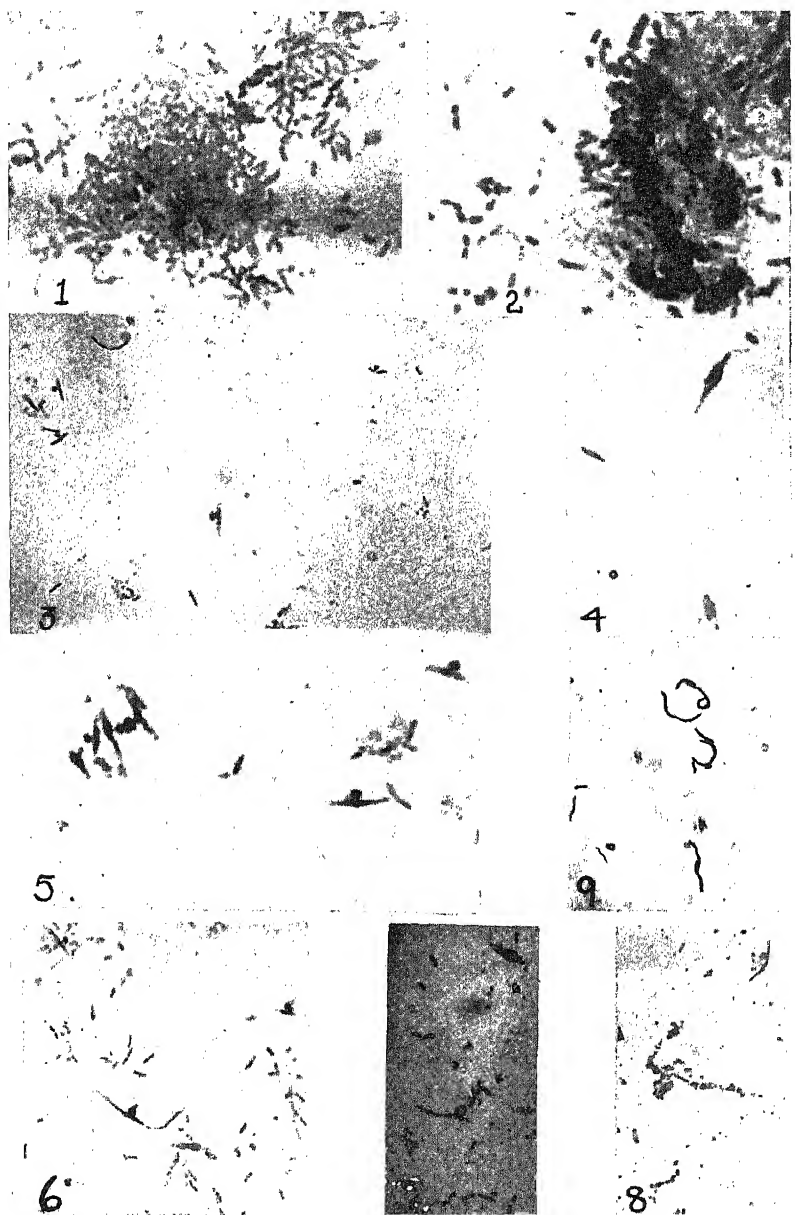
FIG. 4. *Ascobacillus heterozygeus*, from four-month-old pH 4 broth, showing spindle formed by union of two organisms. $\times 1425$.

FIG. 5. *Ascobacillus heterozygeus*, from four-month-old pH 4 broth culture, showing various stages of conjunction and symplasm formation. $\times 1425$.

FIGS. 6 AND 7. *Ascobacillus heterozygeus*, from three-month-old pH 4 broth, showing spiral organization of chromatin in the long spindle. $\times 760$.

FIG. 8. *Ascobacillus heterozygeus*, from three-month-old pH 4 broth, showing symplasm. $\times 760$.

FIG. 9. *Vibriothrix polymorpha*, from forty-eight hour broth culture, showing lateral conjunction of similar organisms. $\times 760$.



(J. Broadhurst, Y. Moriyama and J. Pease: Conjugation of dissexual forms in *Asciobacillus*.)

THE STAINING OF FLAGELLA OF BACTERIA, WITH SPECIAL REFERENCE TO MORDANTS

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Received for publication November 13, 1930

Many mordants have been used in the process of staining bacterial flagella. Among the chemicals most frequently employed in mordants are chromic acid, copper sulphate, ferrous sulphate, ferric chloride, mercuric chloride, osmic acid, potash alum, tartar emetic, tannic acid, basic fuchsin and gentian violet. Casares-Gil's mordant contains the chlorides of aluminum and zinc. Wright (1928) substituted chlorides of cobalt, copper, magnesium, manganese, mercury, nickel and zinc for ferrous sulphate in Loeffler's mordant with very poor results. Ziehl-Neelsen's carbol-fuchsin has probably been used most frequently for staining. Usually mordants have been prepared cold and allowed to stand for some time to "ripen." Heating has often been resorted to in mordanting. Most mordants contain considerable dye and it is probable that many of the best ones contain much material in a colloidal state.

CHEMICALS AND SOLUTIONS USED

In 1929 the writer published a method of staining bacterial flagella. Since then he has tried many mordants in an attempt to find a better one. The following chemicals were used in their preparation: aluminum chloride ($\text{AlCl}_3 \cdot 12\text{H}_2\text{O}$) and silver nitrate from the J. T. Baker Chemical Company; antimonous chloride (SbCl_3), cobalt chloride (CoCl_2), cupric chloride (CuCl_2), ferric chloride (FeCl_3), nickel chloride (NiCl_2), stannous chloride (SnCl_2), zinc chloride (ZnCl_2) and tannic acid from the Merck Chemical Company; basic fuchsin, Coleman and Bell. In addition chromic chloride, lead acetate, lead chloride, manganese

chloride, mercuric chloride and tartar emetic were used, but unsuccessfully. All solutions and mordants were prepared with distilled water in Pyrex, Erlenmeyer flasks of 150 cc. capacity. Percentage solutions were made by adding the weight of chemical indicated by the per cent in grams to 100 cc. of distilled water. For example, a 20 per cent solution contained 20 grams of chemical added to 100 cc. of water.

Two colloidal sols were used in some of the mordants: (1) a ferric hydroxide sol prepared by adding slowly 27.5 cc. of 30 per cent ferric chloride solution to 100 cc. of vigorously boiling water, the boiling continuing till all the ferric chloride had been added; (2) a silver sol, prepared by adding ammonium hydroxide, drop by drop, to 5 cc. of a 1 per cent solution of silver nitrate until the precipitate formed just disappeared; this was diluted with 100 cc. of distilled water and then added slowly, with constant shaking, to an equal volume of a 0.5 per cent tannic acid solution. A similar sol, which proved excellent in mordant no. 22 below, was made by adding the ammoniacal silver nitrate solution to *boiling* tannic acid. These sols are quite stable and will keep several weeks.

The tannic acid solutions were always filtered as soon as made and used *fresh*. The filtered solutions of the chlorides mentioned may be kept as stock solutions and used over a considerable period of time. Saturated solutions of SnCl_2 and SbCl_3 , a 30 per cent solution of FeCl_3 , and 20 per cent solutions of all other chlorides were used. Alcoholic basic fuchsin contained 10 grams of the dye in 100 cc. of 95 per cent alcohol.

MORDANTS

The first satisfactory mordant prepared contained 20 cc. of tannic acid (10 per cent), 6.5 cc. of ferric hydroxide sol and 10 drops of alcoholic fuchsin. The solution of tannic acid was heated to boiling over an asbestos pad. The ferric hydroxide sol was added rather slowly (thirty seconds to one minute) and then the alcoholic fuchsin, drop by drop. Boiling was continued two minutes and then the mordant was cooled and filtered. This mordant was best when one to three days old and gave very good results.

Later it was found that one could substitute 2 cc. of 30 per cent ferric chloride for the ferric hydroxide sol, the procedure for preparation being the same as above. This mordant in some respects was better than the first.

These results suggested trying other chemicals in the same way. Excellent mordants were prepared by substituting solutions of AlCl_3 , CuCl_2 , NiCl_2 and ZnCl_2 for the ferric hydroxide sol. CoCl_2 , SbCl_3 and SnCl_2 were much less satisfactory, while CrCl_2 , lead acetate, PbCl_2 , MnCl_2 , HgCl_2 and tartar emetic could not be used as substitutes for the ferric hydroxide sol.

The fact that Casares-Gil's mordant contains both AlCl_3 and ZnCl_2 suggested substituting combinations of two inorganic salts for the single one in the mordants previously prepared. Of the thirty-six possible combinations in pairs of the chlorides of Al, Co, Cu, Fe, Ni, Sn and Zn and of the ferric hydroxide sol, all but two, CoCl_2 plus NiCl_2 and NiCl_2 plus SnCl_2 , were tried. The six combinations containing CoCl_2 and the six containing SnCl_2 were unsatisfactory or, in most cases, entirely worthless. The best of these, if used fresh or not more than one day old, were CoCl_2 combined with CuCl_2 , FeCl_3 or ZnCl_2 , and SnCl_2 combined with AlCl_3 . All combinations of the chlorides of Al, Cu, Fe, Ni and Zn and of the ferric hydroxide sol, fifteen in number, proved very good to excellent. All pairs containing AlCl_3 or FeCl_3 were excellent as was CuCl_2 plus ZnCl_2 . Those containing NiCl_2 were not quite so good as some others, while CuCl_2 plus ferric hydroxide sol was the poorest.

Besides those already mentioned, three other very good mordants were prepared. These contained alcoholic fuchsin and FeCl_3 , AlCl_3 and ferric hydroxide sol used singly as above; but instead of tannic acid alone a mixture of equal volumes of tannic acid and silver sol. The silver sol was also used in preparing several of the other mordants, but they were more satisfactory without it.

The entire list of mordants is given in tabular form (table 1) and also the general procedure for their preparation.

When SnCl_2 or SbCl_3 was used in mordants only 3 or 4 drops of saturated solutions were added to 20 cc. of tannic acid, while of CoCl_2 4 cc. of a 20 per cent solution were added.

The preparation is practically the same for all of these mordants. The tannic acid, or else the tannic acid and silver sol mixed (nos. 22-24), is heated to boiling in a Pyrex flask. The inorganic salt (nos. 1-6 and 22-24), or the two inorganic salts mixed (nos. 7-21) is added, a few drops at a time, as boiling continues. Then the

TABLE I
Formulas for mordants

MORDANT NUMBER	TANNIC ACID, 10 PER CENT	ALCOHOLIC FUCHSIN, 10 PER CENT	AlCl ₃ , 20 PER CENT	CuCl ₂ , 20 PER CENT	FeCl ₃ , 30 PER CENT	Fe(OH) ₃ SOL	NiCl ₂ , 20 PER CENT	ZnCl ₂ , 20 PER CENT	SILVER SOL
	cc.	drops	cc.	cc.	cc.	cc.	cc.	cc.	cc.
1	20	10	4						
2	20	10		4					
3	20	10			2				
4	20	10				6.5			
5	20	10					4		
6	20	10						4	
7	20	10	2	2					
8	20	10	2		1				
9	20	10	2			3.25			
10	20	10	2				2		
11	20	10	2					2	
12	20	10		2	1				
13	20	10		2		3.25			
14	20	10		2			2		
15	20	10		2				2	
16	20	10			1	3.25			
17	20	10			1		2		
18	20	10			1			2	
19	20	10				3.25	2		
20	20	10				3.25		2	
21	20	10					2	2	
22	10	10	4						10
23	10	10			2				10
24	10	10				6.5			10

alcoholic fuchsin is added and the mixture boiled two minutes longer. After cooling the mordant should be filtered (I use Whatman no. 1 filters). Refiltering on successive days, just before using, is generally necessary.

These mordants are generally most satisfactory when one to four days old, with the exception of nos. 22-24, which are best

when a few hours or a day old. After two to five days they usually fail to work but may be "renewed" by bringing to the boiling point and adding 1 cc. of inorganic salt or salts and 2 drops of alcoholic fuchsin per 10 cc. of mordant. "Renewed" mordants are sometimes better than when first made. They should be filtered after standing a day and on succeeding days just before using.

All of these mordants leave some film on the slide when used. This seems necessary for one cannot stain flagella, it seems, when there is no film at all. The thickness of the film depends on the composition of the mordant, its age and the length of time it is allowed to act. Those containing the silver sol (nos. 22-24) give the least film when fresh, but the others give less as they age until none is formed. "Renewed" mordants may give less film than others.

Soon after their preparation (fresh to one day old) some of these mordants may not require more than one and one-half to two minutes for mordanting, but when older (two to three days) they generally require at least ten minutes. Those containing AlCl_3 , excepting no. 22, or ZnCl_2 do not work when fresh. Apparently all of these mordants contain considerable material in a colloidal state. After standing four or five days a heavy precipitate forms and they often fail to work; i.e., they no longer form a film on the slide. They are always heated when prepared but not for mordanting. They differ from many other mordants in containing less dye. Some dye is necessary but not much. Any one of them may be prepared in a few minutes as the only solution that needs to be fresh is the tannic acid. Large numbers of stained preparations would have to be compared to determine their relative merits. For general use nos. 1, 3 and 22 would doubtless prove sufficient and very satisfactory. However, it is interesting to know that all mordants in this list gave excellent results with the organisms used in this work.

CULTURES

For most of the stained slides of flagella, mixed cultures of bacteria were used. The cultures were prepared by putting one or

two beans in a preparation dish of 200 to 250 cc. capacity, covering with about 150 cc. of tap water and allowing to stand one to two days. Excellent results were also obtained with material taken directly from the teeth and from pure cultures on agar slopes of the following: *Bacillus carotovorus*; *B. cereus*; *B. megatherium*; *B. mycoides*; *B. phytophthorus*; *B. tumefaciens*; *B. vulgatus*; *Pseudomonas campestris*; *Ps. fluorescens*; *Ps. pyocyanea*; *Spirillum rubrum*; and an unidentified organism isolated from diseased Geranium leaves.

Clean, flamed slides were used. On each, four small drops of water (4 to 6 mm. across) were placed, arranged about 1 cm. apart in the form of a square. If bacteria for staining were taken from the mixed cultures mentioned above, two drops of water were placed on a slide, a bean touched to the water until slight cloudiness appeared and then the slide was allowed to stand one to two minutes. A loopful (loop 2 mm. in diameter) of the bacterial suspension was then transferred to the four drops of water on a slide, touching each in succession. This gives four dilutions. The slide was then dried at room temperature in the absence of air currents. When pure cultures on agar slopes were used the procedure was practically the same. A bit of bacterial growth was transferred to two drops of water on a slide, but not mixed with the water; and after two to five minutes a loopful from the edge of the water was touched to four droplets of water on another slide as described above. Sometimes the bacteria were allowed to diffuse, as is so commonly done, for ten to thirty minutes in 5 to 10 cc. of water in a test tube before transferring to the water drops on a slide; but this procedure was generally much less satisfactory than the preceding one. Apparently many flagella break off when the bacteria diffuse for a considerable time in a rather large volume of water. Of course the usual precautions for obtaining actively motile bacteria were observed. Daily transfers were made to nutrient broth, or to nutrient broth and agar slopes alternately, and finally to agar slopes. Best results were generally obtained with cultures about twenty-four hours old; but with some kinds of bacteria (*Bacillus carotovorus*; *B. phytophthorus*; *B. tumefaciens*; *B. vulgatus*; *Pseudomonas*

campestris; *Ps. fluorescens*; *Ps. pyocyanea*) cultures two or three days old were as satisfactory as younger ones. Excellent preparations of *Ps. pyocyanea* and *Ps. campestris* were made from cultures seven days old. No other cultures so old as this were tried. In all cases if the bacteria were *actively motile* the flagella could be stained readily. It is very important to have the bacteria quite free from the nutrient medium, especially proteinaceous material, in all staining of flagella. If more than a trace of the medium is present the flagella stain poorly or not at all. Making dilutions in four drops of water on the slide will usually result in at least one, sometimes all, being satisfactory.

STAINS AND STAINING

Although several staining solutions were tested, no other, not even carbol fuchsin, proved as satisfactory as anilin-alcohol-fuchsin (Maneval, 1929). This stain contains:

	cc.
Water, distilled.....	30
Basic fuchsin, 10 per cent alcoholic.....	10
Anilin oil (1 part) and 95 per cent alcohol (3 parts) mixed.....	5
Acetic acid, 4 per cent.....	1

Mix in the order given, filter once or twice and again before using. It is best after three to six days and may sometimes be kept several weeks, but it finally deteriorates. Good results have been obtained by substituting rosanilin or methyl violet for the basic fuchsin in this formula. Anilin fuchsin and anilin gentian violet were poorer.

The air-dried slides were placed on a level surface, treated with 4 to 8 drops of mordant and allowed to stand two to ten minutes. (One or two trials indicated the time to use.) They were washed with 6 to 10 changes of water, being careful to hold them level when the water was first poured on. After the second change of water the preparations were wiped carefully with a clean towel to remove the edge of the film of mordant which may harden somewhat, and upon completing the washing treated with 3 to 5 drops of stain for three to five minutes. After washing thoroughly and drying they were examined with the immersion objective without a cover glass.

If preparations are satisfactory there will always be some film present. The position of the four drops of water may be seen against a white background. Just inside the edge of each drop there will be a clearer zone in which the flagella will generally be stained better than elsewhere. When these mordants are used fresh or a day old the stained flagella usually appear rather thick but thinner if the mordants are older, until finally they will appear scarcely visible. This depends not only on age, but also on composition of the mordant and time used for mordanting. Sometimes the stained film will appear under the microscope as made up of very numerous small droplets. This may indicate that the mordant is not old enough or that it or the stain should be filtered.

It seems very likely that the excellence of the mordants recommended here depends largely on the existence in them of much material in a colloidal state; and that some solutions fail to act as mordants because a suitable sol is not obtained in preparing them. The tannic acid and the sols of $\text{Fe}(\text{OH})_3$ and Ag are colloidal. It is possible that when the various chlorides used successfully are added to boiling tannic acid they pass over into a colloidal condition, the same as FeCl_3 does when added to boiling water. After standing several days heavy precipitates generally form and the efficiency of the mordants is much decreased. It seems probable that staining results mainly from adsorption by the flagella, so that a layer of mordant and dye is formed over them which may make them appear much thicker than they really are. No doubt the size and electrical charge of the colloidal particles, as well as H-ion concentration are important factors in the results obtained, but they have not been investigated experimentally.

SUMMARY

Hundreds of excellent stained preparations of flagella of numerous kinds of bacteria were made. The air-dried "smears" were mordanted in most cases with a solution containing tannic acid, alcoholic basic fuchsin and a chloride of Al, Cu, Fe, Ni or Zn, a ferric hydroxide sol, or else combinations of these chlorides

in twos with each other or with the $\text{Fe}(\text{OH})_3$ sol. Three mordants contained a silver sol. Formulas are given for twenty-four mordants. The stain used was an alcoholic-anilin-fuchsin. It seems probable that the efficiency of these mordants depends largely on their colloidal condition. Their use makes the staining of bacterial flagella relatively easy.

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THE BACTERIOLOGICAL EVALUATION OF SOME NEW WATER SOLUBLE ORGANO-MERCURY COMPOUNDS¹

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Received for publication December 1, 1930

INTRODUCTION

The therapeutic use of organo-mercury compounds as bactericides is dependent in large measure on their solubility in water and their toxicity. Two methods for increasing the solubility of such compounds have been used. Introduction of acid- or nitrogen-containing groups gives increased solubility, but the former cause a loss of germicidal action and the latter give products having much greater toxicity than the acid derivatives. Neither of these procedures has therefore given a satisfactory solution to the problem. A chemical combination which would produce increased water solubility and lower toxicity would do much toward bettering the application of organo-mercury compounds in the therapeutic field.

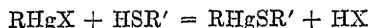
In 1922 Kharasch² developed a method whereby organo-metallic compounds can be made soluble by condensing them with mercapto-carboxylic or sulfonic acids.

When mercurials of the type $RHgX$, where X is an inorganic radical, react with a mercapto acid, HSR' where R' is a group

¹ The chemical preparation of these compounds has been presented in abstract before the Division of Medicinal Products of the American Chemical Society at the Detroit meeting, September, 1927, by M. S. Kharasch, H. A. Shonle and John H. Waldo and at the Atlanta meeting, April, 1930, by John H. Waldo and H. M. Powell. The authors wish to express their appreciation to Drs. G. H. A. Clowes and M. S. Kharasch and Mr. W. A. Jamieson, for advice and suggestions in the conduct of this work.

² U. S. Patent 1,589,599, reissue 16921.

containing an alkyl or aryl carboxylic or sulfonic acid, a double decomposition occurs:



The new compound RHgSR' is soluble in NaHCO_3 solution and forms with the alkali metals soluble salts whose solutions are in general stable and do not give an immediate precipitate of HgS with $(\text{NH}_4)_2\text{S}$.

In collaboration with Kharasch, Cohen (1929) investigated alkyl mercurithioglycollic acids and found them to possess marked bactericidal properties.

In collaboration with Kharasch an extended series of mercury compounds of the general formula RHgSR' were prepared and tested in this laboratory.³ This series might be expected to contain members which would be very active germicides and yet possess the low toxicity of mercury compounds containing carbonyl groups.

In the selection of a substance therapeutically valuable as a germicidal agent, such factors as solubility, stability, lack of marked acid or basic reactions, absence of inhibition of cell growth and low toxicity must be considered. Also effective bactericidal and bacteriostatic action against both vegetative and spore formers especially in the presence of adequate organic matter must be shown. In evaluating a series of germicides, other factors being equal, that compound having maximum destructive effect on bacteria and minimum toxicity would be the most desirable for therapeutic use. For these reasons due attention was given to toxic properties and it was decided to adopt a preliminary standard test, as outlined by Reddish (1927) for comparative bactericidal tests.

To avoid the criticism which might be offered against bactericidal tests in watery media, an additional test, modifying this technique by using normal horse serum as diluent instead of distilled water, has been carried out. It was first determined that the compounds used did not precipitate the proteins of

³ U. S. Patent 1,672,615.

normal serum, a characteristic frequently common to organo-mercury compounds.

BIOLOGICAL EVALUATION

The compounds were first evaluated by the standard Reddish test, and then by the following modification, which consisted in planting 0.5 cc. of twenty-four-hour broth culture into 5 cc. quantities of germicide dilution⁴ made with undiluted normal horse serum. After a ten-minute exposure at 20°C., a 3-mm. loop of this material was subcultured to broth and readings were made after forty-eight hours incubation. Tubes showing no growth at this time produced normal growth when planted with 0.02 to 0.01 cc. of old untreated culture. For comparative purposes, three commercial germicidal preparations were selected and submitted to test by identical technique.



A test with highly virulent organisms was introduced to determine the effect of these germicides on bacteria directly cultured from the blood stream of animals. Pneumococci (type I) of such a virulence that a hundred millionth to a billionth cubic centimeter of a broth culture was fatal to mice were planted in 0.5 cc. doses into 5 cc. quantities of aqueous germicidal dilution. After an exposure of thirty minutes transplants were made into broth and in addition 0.1 cc. quantities were injected into mice. The presence of viable organisms in the germicide mixture was determined by growth in the subculture tubes and by death of the injected mice. There was complete agreement between these two tests. It was noted that organisms directly cultured from an animal are much more resistant to a germicide than organisms passed through several subcultures in media.

It appears that the figures representing the toxicity of organo-mercury compounds should represent not alone the acute or immediate degree of toxicity, but should be representative of any later effects as well. For this reason, toxicity figures recorded in the table were determined after seven days observation, and the M.L.D. was determined from animals surviving after that time. It has been noted that following such a period of observation,

⁴ The original germicides were 1 per cent water solutions.

TABLE 1
Biological tests

COMPOUNDS	TOXICITY (RATS) M.L.D. mgm. per kgm.	DILUTIONS				
		Reddish test		Modified Reddish		Against virulent organisms
		<i>B. typhosus</i>	<i>Staph. aureus</i>	<i>B. typhosus</i>	<i>Staph. aureus</i>	Pneumo- coccus
<chem>CH3HgS-C6H11COOH</chem>	40	1:2000	1:5000	1:2000	1:4000	1:1000—
<chem>C2H5HgS-C6H11COOH</chem>	50	1:4000	1:5000	1:3000	1:4000	1:1000
<chem>iso-C6H11HgS-C6H11COOH</chem>	60	1:2000	1:3000	1:2000	1:3000	1:1000—
<chem>C6H5HgS-C6H11COOH</chem>	15	1:3000	1:2000	1:1000—	1:1000—	1:1000—
<chem>C6H5CH2HgS-C6H11COOH</chem>	*	1:1000—	1:1000—	1:1000—	1:1000—	1:1000—
<chem>C2H5HgS-C6H10COOH</chem>	40	1:2000	1:3000	1:2000	1:3000	1:1000—
<chem>C3H7HgS-C6H10COOH</chem>	100	1:1000	1:1000	1:1000	1:1000—	1:1000—
<chem>C2H5HgS-C6H9COOH</chem>	100	1:1000	1:3000	1:1000	1:2000	1:1000—
<chem>C3H7HgSCH(COOH)CH2CH3</chem>	80	1:1000—	1:1000—	1:1000—	1:1000—	1:1000—

C_2H_3HgS-  $-SO_3H$	25	1:1000—	1:1000	1:1000—	1:1000	1:1000—
C_6H_3HgS-  $-SO_3H$	10	1:1000—	1:1000—	1:1000—	1:1000—	1:1000—
Germicide A.....	10	1:8000	1:6000	1:1000	1:1000	1:2000
Germicide B.....	4	1:10,000	1:6000	1:2000	1:3000	1:1000—
Germicide C.....	50	1:100	1:100—	1:100—	1:100—	1:500—

Note: A minus sign following the indicated bactericidal dilutions means that the effective concentration is somewhat greater. Exposures are for ten minutes at 20°C., in the Reddish and modified Reddish Tests, and for thirty minutes against virulent pneumococci.


* Insufficient material was available to complete toxicity tests.

surviving animals suffer no such ill effects as latent mercury poisoning. Toxicity was determined by intravenous injection, and three well known commercial preparations were used as controls. The data thus obtained are tabulated in table 1.

It was found that in the presence of serum the bactericidal action of these mercurials was not materially lower than their action in the presence of water. This is in distinct contrast to the general observation that the presence of serum tends to reduce the effectiveness of germicides.

Table 1 shows that one member, ethyl-mercuri-thiosalicylic acid, is quite superior in all three properties recorded. It combines the greatest bactericidal action both in the general test

TABLE 2
Bacteriostatic value

GERMIDCE	24 HOURS		48 HOURS		72 HOURS	
	<i>Staph. aureus</i>	<i>B. typhosus</i>	<i>Staph. aureus</i>	<i>B. typhosus</i>	<i>Staph. aureus</i>	<i>B. typhosus</i>
C_2H_5HgS —  —COOH.....	1:3,000,000	1:1,000,000	1:2,000,000	1:700,000	1:1,000,000	1:700,000
HgCl ₂	1:100,000	1:1,150,000	1:50,000	1:100,000	1:50,000	1:50,000
Germicide B.....	1:1,000,000	1:50,000	1:700,000	1:50,000	1:500,000	1:50,000

and against virulent organism with a low degree of toxicity. It has also been demonstrated to possess a minimum hemolytic action toward red blood cells. This work will be more specifically outlined in conjunction with more extensive tests and will be reported upon at a later date.

For this reason bacteriostatic tests were carried out on the above product, and comparison made with mercuric chloride and a commercial mercurial germicide. A 3-mm. loop of twenty-four-hour broth culture was planted into 5 cc. quantities of the indicated dilutions of germicide made in broth and incubated at 37.5°C. Readings for growth were made at the end of twenty-four, forty-eight and seventy-two hours.

SUMMARY

1. The animal toxicity and bacteriostatic and bactericidal action of some new organo-mercury germicides has been presented.

2. One of the group, ethyl-mercurithiosalicylic acid has been shown to possess properties indicating particular value as a germicide.

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BACTERIAL SPORES¹

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Received for publication December 3, 1930

INTRODUCTION

According to Teasi Matzuschita (1902) bacterial spores were first recognized and described by Pertz in 1852 in a book entitled "Zur Kenntniss kleinster Lebensformen." Since the discovery of *B. anthracis* the nature of bacterial spores and the conditions under which they are formed have been extensively studied. A number of organisms, including *Bact. typhosum* and *M. tuberculosis*, were for a time believed to form spores. This has now definitely been disproved. Heat resistance of a culture has become the generally accepted criterion of the presence of spores. Spores generally have a thermal death point 30° or 40°C. higher than that of vegetative cells.

It seems quite definitely proved that sporulation may occur in any spore forming bacteria irrespective of the number of generations they are removed from the parent spores. According to Garbowsky (1907) the very first organisms which developed from a spore are capable of sporulating. Schreiber (1896) has substantiated this observation. Concerning pre-spore changes which take place in the organisms there seem to be considerable differences of opinion. Many of the early investigators believed that the spore resulted from a condensation of large pre-spore granules. Subsequent work, however, especially by Schaudinn (1902), Guilliermond (1910), and Schwellengrebel (1913), shows that

¹ Part of a dissertation submitted to the Department of Hygiene and Bacteriology of the University of Chicago to fulfil certain requirements for the degree of Doctor of Philosophy.

such granulation does not always take place. These investigators find that at the time of sporulation chromatin granules appear in the cells. These granules arrange themselves in certain patterns and together with other constituents of the cell condense to form the spore. A great deal has been written about the presence of nuclei in spore forming bacteria and their relation to sporulation. No definite agreement or conclusions seem to have been reached, however. Bunge (1895), and Grethe (1897) first expressed the idea that the proteins of the vegetative cells and of the spores are different. Later workers, including Ruzicka (1913), and Mellon and Anderson (1919) have expressed the same idea. Chemical analysis of spores seem to show that they consist mostly of proteins. Cramer (1894) found the protein content to be 70 to 90 per cent

Extensive investigations have been made of the properties of spores, and especially their resistance to heat and chemicals. The conditions under which they are stored seem to have an effect on their resistance to heat and chemicals. Some of the later investigations on the effect of heat are those of Burke (1923), Bigelow (1921), Reiter (1920), Esty and Meyer (1922), Magoon (1926), and Williams (1929). It seems definitely established that certain media produce spores of higher thermal resistance than other media. As to the effect of age and conditions of storage there seems to be a lack of agreement. The effect of chemicals has been extensively studied, especially by Müller (1920) and Dozier (1924). It seems that under certain conditions chemicals which inhibit sporulation lead to the production of asporogenic strains of the organisms. *B. anthracis* seems to form asporogenic strains most readily.

The conditions most favorable for sporulation have been found to be essentially those most favorable for growth, except for the absence of food (Buchner (1890), Weil (1899), Matzuschita (1902)). The effect of oxygen has been extensively studied by Meyer (1909) and Bredeman (1909). In general, the optimum oxygen concentration for the sporulation of aerobes seems to be atmospheric concentration, and for that of anaerobes close to 0. The optimum temperature for sporulation corresponds closely

with that for growth. The limiting temperature for the sporulation of most of the ordinary bacteria is between 15° and 45°C. A good review and discussion of this subject is given by Holzmüller (1909).

OBSERVATIONS FROM EXPERIMENT

Hydrogen-ion concentration and sporulation

The hydrogen-ion concentration of a medium has a considerable influence on sporulation. The idea seems to have become general that an alkaline medium is more favorable for sporulation than an acid one. The experience of the author has been that a neutral or slightly acid medium is more favorable for sporulation than an

TABLE 1
Sporulation at different hydrogen-ion concentrations

MEDIUM NUMBER	Na ₂ HPO ₄ (0.3M)	NaH ₂ PO ₄ (0.3M)	pH FINAL	SPORES			
				<i>Cl. botulinum</i>	<i>Cl. tetani</i>	<i>B. vulgatus</i>	<i>B. circulans</i>
				per cent	per cent	per cent	per cent
1	0	1.0	5.1	No growth	2	80	No growth
2	0.05	0.95	5.6	No growth	10	90	?
3	0.15	0.85	6.0	90	40	90	<1
4	0.20	0.80	6.2	90	80	90	10
5	0.30	0.70	6.4	50	40	90	5
6	0.90	0.1	8	<1	5	20	<1
7	0.95	0.05	8.4	<1	2	40	<1

alkaline one. It is not so much the initial pH of the medium (nor the final one) which is important but the pH at the time of sporulation.

A number of experiments were performed to determine the optimum hydrogen-ion concentration for the sporulation of *Cl. botulinum*, *Cl. tetani*, *B. vulgatus*, and *B. circulans*. More experiments were made with *Cl. botulinum* than with the others. The medium used was 2 per cent agar in slants with 1 per cent peptone, 0.5 per cent beef extract, and the buffer mixtures as indicated. The cultures were all incubated at 37°C. and the anaerobes in an anaerobic jar. A typical result is given in table 1.

It is concluded from these experiments that for *Cl. botulinum*, *Cl. tetani*, *B. vulgatus*, and *B. circulans* the optimum hydrogen-ion concentration for sporulation on phosphate buffered extract agar is distinctly acid and at a pH of about 6.2.

Effect of carbohydrates on the sporulation of Cl. botulinum

The presence of a fermentable sugar in a medium generally inhibits sporulation. The cause of this inhibition may be (1) the acid produced, (2) the carbon dioxide produced, or (3) the carbohydrate as such. Non-fermentable sugars have no inhibiting effect on sporulation. *Cl. botulinum*, for example, will grow and sporulate in 60 per cent sucrose.

TABLE 2

Effect of various concentrations of glucose on the sporulation of Cl. botulinum

MEDIUM NUMBER	GLUCOSE	pH		pH CHANGE	SPORES
		Initial	Final		
	<i>per cent</i>				
1	1	6.87	5.35	-1.48	—
2	0.8	—	5.53	—	—
3	0.6	6.98	5.61	-1.37	—
4	0.4	7.00	5.93	-1.07	—
5	0.2	7.00	6.49	-0.51	+
6	0.1	7.00	6.55	-0.45	++
7	0	7.00	6.85	-0.15	++

A number of experiments were performed to determine the effect of glucose on the sporulation of *Cl. botulinum*. To a basic medium of extract bouillon were added varying amounts of glucose. The results after anaerobic incubation at 37° for four days are given in table 2.

It would seem from table 2 that whenever sufficient acid is produced to bring the pH of the medium below a certain value no sporulation occurs.

The limiting hydrogen-ion concentration for the sporulation of *Cl. botulinum* was previously found to be about pH 6.0. This would tend to indicate that the acid produced by the fermentation of the sugar is responsible for the inhibition of sporulation.

TABLE 3

Effect of various salts on growth and sporulation of Cl. botulinum and Cl. tetani

SALT	CONCENTRATION	pH INITIAL	CL. BOTULINUM		CL. TETANI	
			Growth	Spores	Growth	Spores
	<i>per cent</i>					
Na ₂ HPO ₄	4	7.9	+	+	+	+
	8		+	+	+	+
	12		+	—	+	+
	16		+	—	+	+
	20		—		—	
Na ₂ SO ₄	2	7.8	++	—	+	+
	4		++	+	+	+
	6		++	+	+	+
	8		++	+	+	+
	10		+	A few	+	+
NaCl.....	3	7.6	++	—	++	++
	6	7.7	+	—	—	
	9		—		—	
Na-citrate.....	4	7.8	+	+	—	
	8		—			
Na-oxalate.....	0.4	7.7	+	A few	+	+
	0.8	7.9	+	+	+	+
	1.2		+	+	+	+
	1.6	8.1	+	—	—	
	2.0		—		—	
K-phthalate.....	2	7.8	+	—	+	++
	4		+	++	+	—
	6		+	+	+	—
	8		+	—	Poor	—
	10	8.1	+	—	?	—
K ₂ SO ₄	2	7.8	+	+	+	+
	4		+	++	+	+
	6		+	+	+	+
	8		+	A few	+	+
	10		Poor	—	+	A few
KCl.....	3	7.5	+	—	+	++
	6		Poor	—	+	—
	9		—		Poor	—
	12				—	

TABLE 3—Continued

SALT	CONCENTRATION	pH INITIAL	CL. BOTULINUM		CL. TETANI	
			Growth	Spores	Growth	Spores
	<i>per cent</i>					
MgSO ₄	2.4	7.4	+	A few	+	+
	4.8		+	A few	+	++
	7.2		—		+	+
	9.6				+	+
	12				+	Poor
MgCl ₂	2	7.1	+	+	+	++
	4		Poor	—	+	+
	6		—		?	—
Ca(NO ₃) ₂	0.2	6.8	+	—	+	+
	1		+	—	+	+
	3		+	—	+	+
	6		—		—	
CaCl ₂	1	6.7	+	—	+	+
	2		—		+	—
	3				—	
Ca-lactate.....	2	6.8			+	+
	4		+	+	+	+
	6		+	+	+	+
	8		+	A few	+	+
	10		—		—	
BaCl ₂	0.5	6.9	+	—	+	+
	1		—		+	—
	2				—	
Pb-acetate.....	0.008	7.2	+	—	+	+
	0.04		+	—	+	+
NH ₄ Cl.....	0.8	7.0	+	+	+	++++
	1.6		+	++	+	++++
	2.4		+	++	+	++++
	3.2	6.7	—		+	++
	4.0				—	
NH ₄ -phosphate.....	2	7.1	+	+++	+	+++
	4		+	++	—	
	6		—			

TABLE 3—Concluded

SALT	CONCENTRATION	pH INITIAL	CL. BOTULINUM		CL. TETANI	
			Growth	Spores	Growth	Spores
	<i>per cent</i>					
NH ₄ -citrate.....	1	7.4	+	+	+	++
	2	7.2	—		—	
NH ₄ -oxalate.....	0.5	7.4	+	+	+	++
	1	7.4	—		—	
NH ₄ -sulphate.....	2	6.9	+	+++	+	+++
	4		—		+	++
	6	6.8			—	
NH ₄ Br.....	1	7.3	+	++	+	++
	2		+	++	+	+++
	3		+	++	+	++
	4		+	+	+	+

Effect of inorganic salts on sporulation

The above table 3 gives the effects on sporulation of *Cl. botulinum* and *Cl. tetani* of a number of inorganic salts when added to a basic medium consisting of 1 per cent peptone, and 0.5 per cent beef extract. The tubes were incubated anaerobically for seven days. Table 3 shows that almost invariably growth occurs at a higher concentration than sporulation. Chlorides and nitrates prevent sporulation of *Cl. botulinum* but not to the same extent as in the case of *Cl. tetani*. Ammonium salts and sulphates stimulate sporulation of both organisms.

Chemical elements necessary for sporulation

Extract bouillon itself has all the ingredients necessary for some sporulation. The effect of added salts, whether stimulating or inhibiting, may be either chemical, physical, or both. If the salts are added to a basic medium consisting of peptone only, a better understanding of the effect of the salt may perhaps be obtained. One such experiment was performed. The composition of the media and the results, after five days of anaerobic incubation, are given in table 4.

The media in table 4 which show sporulation in both instances are nos. 3, 10, and 13. All three of these media contain both ammonium and phosphate ions. None of the other media except 15 have both of these ions. Medium 9 showed a few spores in the first set but not in the second. This medium contained ammo-

TABLE 4

Sporulation of Cl. botulinum in various media containing 1 per cent peptone plus various salts as indicated

MEDIA	SALTS ADDED	pH	FIRST SET		SECOND SET	
			Growth	Spores	Growth	Spores
				per cent		per cent
1		7.9	++	—	++	—
2	NH ₄ Cl—0.2M	7.3	+	—	+	—
3	(NH ₄) ₂ HPO ₄ —0.2M	6.2	—	—	—	—
	(NH ₄) ₂ HPO ₄ —0.2M	7.2	++	10	++	5
4	(NH ₄) ₂ SO ₄ —0.2M	6.4	—	—	+	—
5	NaH ₂ PO ₄ —0.2M	6.0	—	—	—	—
	Na ₂ HPO ₄ —0.2M	7.9	++	—	++	—
	Na ₃ PO ₄ —0.2M	8.5	+	—	+	—
6	K ₂ SO ₄ —0.2M	8.1	+	—	+	—
7	CaCl ₂ —0.02M	7.8	++	—	++	—
8	MgCl ₂ —0.02M	8.0	++	—	++	—
9	NH ₄ Cl—0.2M + CaCl ₂ —0.02M	7.3	+	1	+	—
10	(NH ₄) ₂ HPO ₄ —0.15M + CaCl ₂ —0.05M	6.1	—	—	—	—
	(NH ₄) ₂ HPO ₄ —0.15M + CaCl ₂ —0.05M	6.9	++	20	++	15
11	(NH ₄) ₂ SO ₄ —0.15M + MgCl ₂ —0.05M	7.3	+	—	+	—
12	Na ₂ HPO ₄ —0.15M + MgCl ₂ —0.05M	7.7	++	—	++	—
13	(NH ₄) ₂ SO ₄ —0.1M + Na ₂ HPO ₄ —0.1M + MgCl ₂ —0.05M	6.9	++	1	++	1
14	NaH ₂ PO ₄ —0.1M + K ₂ SO ₄ —0.1M	6.6	++	—	++	—
	Na ₂ HPO ₄ —0.1M + K ₂ SO ₄ —0.1M	7.1	++	—	++	—
	Na ₃ PO ₄ —0.1M + K ₂ SO ₄ —0.1M	8.4	+	—	+	—
15	(NH ₄) ₂ HPO ₄ —0.15M + MgCl ₂ —0.05M	6.9	+	—	+	—

nium and calcium ions. It seems that ammonium and phosphate ions are essential for the sporulation of *Cl. botulinum*. Beef extract may be somewhat deficient in these ions and hence the stimulating effect on sporulation when they are added to extract bouillon. The calcium ion also seems to have some stimulating effect.

Effect of oxygen on sporulation

Sporulating organisms seem to be either strict aerobes or strict anaerobes. The optimum concentration of oxygen for the sporulation of aerobes has been extensively studied in the past by Meyer (1909) and others. In the case of strict anaerobes the optimum oxygen concentration for sporulation is very close to

TABLE 5
Effect of oxygen on the sporulation of a number of aerobes and anaerobes

ORGANISMS	OXYGEN TENSION OF MERCURY					
	0 cm.*		1 cm.		2 cm.	
	Growth	Spores per cent	Growth	Spores per cent	Growth	Spores
<i>Cl. botulinum</i> A.....	+++	95	+	10	0	
<i>Cl. botulinum</i> B.....	+++	80	+	10	0	
<i>Cl. chauveii</i>	+++	90	+	90	+	0
<i>Cl. sporogenes</i>	+++	90	+	90	+	0
<i>Cl. tetani</i> (atypical).....	+++	95	+	50	0	
<i>Cl. tetani</i>	++	30	+	0	0	
<i>Cl. novyi</i>	+	2	0	0	0	
<i>Cl. oedematiens</i>	+++	50	+	60	0	
<i>Cl. septicum</i>	+++	95	+	40	+	0
<i>B. anthracis</i>	0		0		++	0
<i>B. vulgatus</i>	0		1 colony?	30	+++	?
<i>B. circulans</i>	+	0	++	0	+++	0
<i>B. cereus</i>	0		0		+	0

* This oxygen tension was undoubtedly greater than zero. Just what it was is impossible to say. The agar was freshly boiled. The nitrogen was passed through a long column of yellow phosphorus before it was admitted to the anaerobic jar. The water in the bottom of the jar was allowed to boil under the vacuum for some time. Any oxygen left must certainly have been at a very low concentration.

zero. Table 5 gives the results of a number of experiments on this subject. The organisms were grown on agar slants in anaerobic jars which had first been freed of oxygen by alternately evacuating and filling with nitrogen and then the desired amount of air let in as measured by a manometer. The aerobes and anaerobes were kept in separate jars. The cultures were incubated for four days.

It may be observed from table 5 that the anaerobes show a considerable difference in their ability to sporulate under increased oxygen tensions. The sporulation of *Cl. tetani* and *Cl. novyi* was inhibited at 1 cm. of oxygen. All the others except *Cl. sporogenes* and *Cl. chauweii* sporulated less at 1 cm. of oxygen than at 0 cm. (see note). The sporulation of all the organisms was inhibited by 2 cm. of oxygen. In the case of the aerobes no sporulation was observed at 2 cm. of oxygen although fair growth was obtained. *B. circulans* seems to be the most "anaerobic" of the aerobes used—growing as it did in less than 1 cm. of oxygen.

Most strict aerobes sporulate poorly in bouillon, mainly because of the low oxygen concentration. It has been my practice to place a freshly inoculated slant of *B. subtilis* or *B. anthracis* in my anaerobic jars as a check on the anaerobic condition. If the aerobe does not grow, the air tension is probably below 1 cm. of mercury as indicated by my own work and that of others. If it grows but does not sporulate, the air tension is probably between 1 and 4 cm. of mercury.

Experiments with Cl. botulinum

The changes which take place in the properties of an organism as it passes through its spore cycle have ordinarily been studied separately. In the following pages are presented the results of some experiments with *Cl. botulinum* type A, in which the change of a number of properties was studied simultaneously as the organism passed through its spore cycle. The following changes of the organisms and the medium were determined: General morphology of the organisms, pH of the medium, cataphoretic potential of the organisms, oxygen sensitivity of the organisms, thermal death time-temperature of the organisms, and toxicity of the medium.

Culture medium

The experiments, first of all, demanded a medium in which the organisms could be made to germinate and sporulate within definite time limits, and in which the sporulation would be at least 90 per cent. Such a medium had been developed earlier in

the work. The medium consists of 1 per cent peptone (Wilson), 0.5 per cent beef extract (Swift), 0.5 per cent Na_2PO_4 , + 0.5 per cent $(\text{NH}_4)_2\text{SO}_4$. No adjustment of reaction was made, and it was found to vary from pH 6.7 to pH 6.9. For the nutrient agar, 2 per cent agar was added. In this medium germination occurred in from fifteen to twenty hours and sporulation in from forty to forty-eight hours. The degree of sporulation varied from 90 to 100 per cent.

Anaerobic jars

The next requirement was a number of anaerobic jars which could be handled with ease, and which would allow regular growth on agar slants. A very convenient and inexpensive anaerobic jar was made as follows: Into empty peptone jars (or similar glass jars) were placed two-hole rubber stoppers. Into one hole was placed a mercury manometer and into the other a two-way stopcock, as shown in figure 1. In the bottom of each jar was placed a small quantity of pyrogalllic acid (3 to 5 grams), and a few grams of Na_2CO_3 . Na_2CO_3 was used in place of NaOH because of the latter's affinity for CO_2 . The presence of a strong solution of NaOH was found to inhibit growth on agar slants. The rubber stopper was greased with a mixture of vaseline and beeswax, and the stopcock with a heavy rubber grease. At incubator temperature (37°C .) ordinary vaseline and stopcock grease are too liquid to be efficient. When the tubes were in the jar a little water was added by means of a pipette, and the jar evacuated with a water pump. Connection with a nitrogen tank was made by one arm of the stopcock. As the vacuum was being built up a little nitrogen was run in to remove the air in the arm of the stopcock. The jar could then be filled with pure nitrogen after evacuation by the water pump. To facilitate removal of the oxygen the water in the bottom of the jar was kept warm so that it boiled under the vacuum. The nitrogen was freed of oxygen by passing it through a long column of yellow phosphorus, followed by several bottles of alkali to remove the P_2O_5 formed. Commercial nitrogen seems to contain a considerable amount of oxygen and is

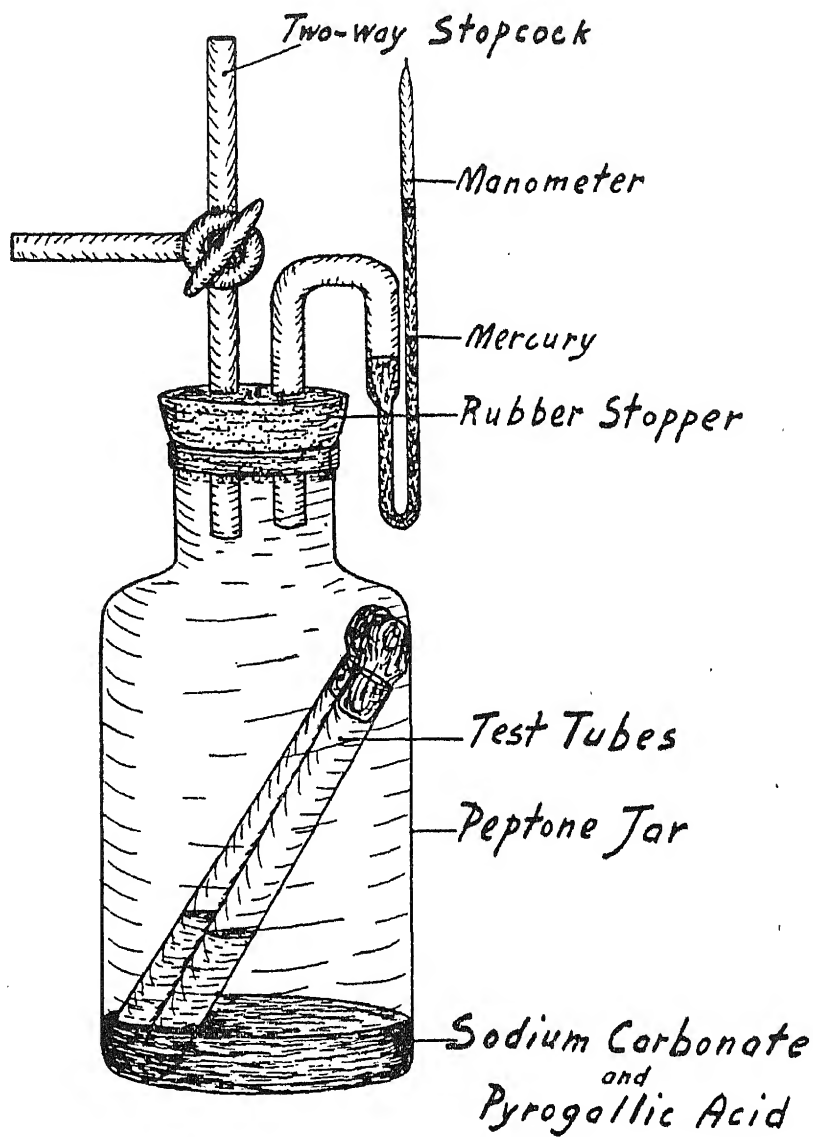


FIG. 1. A SIMPLE AND INEXPENSIVE ANAEROBIC JAR

not to be recommended for use in anaerobic work. Commercial hydrogen is much purer.

Method of conducting the experiment

A number of freshly slanted agar slants were inoculated with *Cl. botulinum* type A, and incubated at 37°C. for four days. The jar was then opened and the slants left in the incubator for one day. This exposure to air at 37°C. was found to disintegrate completely the vegetative part of the cells in which spores had formed. The spores were then removed from the slants, washed by centrifuging, suspended in bouillon, and heated at 70°C. for ten minutes to destroy any toxin they might contain. About 30 freshly boiled tubes of bouillon were inoculated at 50°C. from the spore suspension by means of a pipette. Four tubes of bouillon were placed in each of a number of anaerobic jars and incubated at 37°C. At different intervals of time, so chosen that the organisms would be in distinctly different developmental stages, the different jars were opened and the examination made. As soon as a jar was opened the tubes were placed in ice water to prevent further change.

Changes in morphology of the organisms

In the germination of *Cl. botulinum* the new vegetative cell comes out of the end of the spore opposite that to which the old vegetative cell is or was attached. The first sign of germination is swelling and increased affinity for stain. The vegetative cell then grows out of one end of the swelled spore. A great number of spores always fail to germinate. Germination was generally complete in from fifteen to twenty hours. In thirty-five to forty hours signs of sporulation began to appear as highly stainable swellings close to the end of the organism. These swellings gradually lost their affinity for stain and sporulation was complete in forty to forty-eight hours. The swollen spore preceding germination, the very first bud of vegetative cell coming out of the spore, and the swelling of the young spore were all intensely Gram positive.

Changes in pH of the medium

The pH determinations were made electrometrically by means of the quinhydrone electrode. The electrode vessel was made by cutting off two test tubes 2 inches from the bottom and sealing a stopcock between them. The stopcock formed a bridge between the two tubes. The whole apparatus was filled with the liquid to be tested. Quinhydrone and a gold electrode were placed in one tube

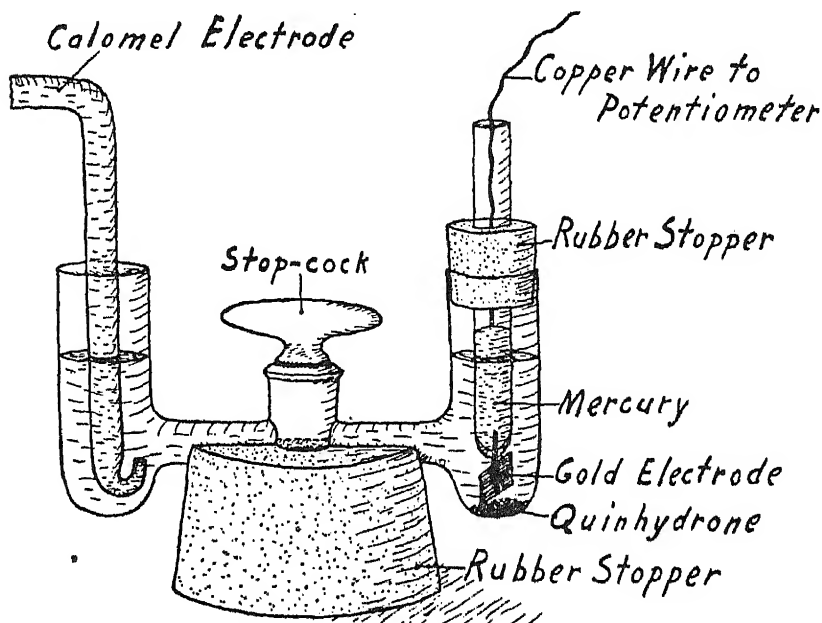


FIG. 2. APPARATUS FOR DETERMINING HYDROGEN-ION CONCENTRATIONS ELECTRO-METRICALLY BY MEANS OF THE QUINHYDRONE ELECTRODE

of the apparatus, and the arm of a calomel cell in the other. A drawing of the apparatus is shown in figure 2. From three of the spore cycles a pH reading was taken from every jar. These three readings were averaged and the resulting curve is shown in figure 3. A change of pH of about 0.2 is noted at the time of sporulation. In a medium as well buffered as this one, the change is of some significance.

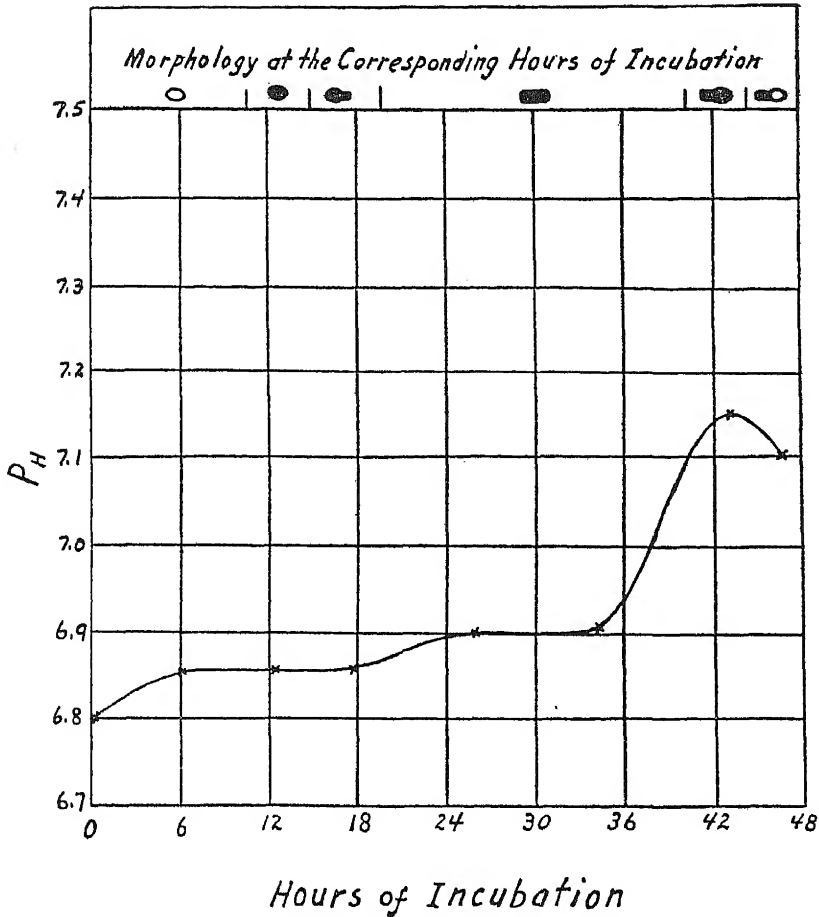


FIG. 3. AVERAGE HYDROGEN-ION CONCENTRATIONS OF THE *CL. BOTULINUM* CULTURES AFTER VARIOUS PERIODS OF INCUBATION

Changes in cataphoretic potential of the organisms

The data for this experiment were obtained by means of an apparatus with non-polarizable electrodes, somewhat similar to that originally described by Northrop. No attempt was made to standardize it since only relative values were desired. In each case the organisms were centrifuged and resuspended in the origi-

nal bouillon. The readings on the young vegetative cells are poor because of their motility. The readings of the different cycles are averaged and plotted in figure 4. The migration velocity is

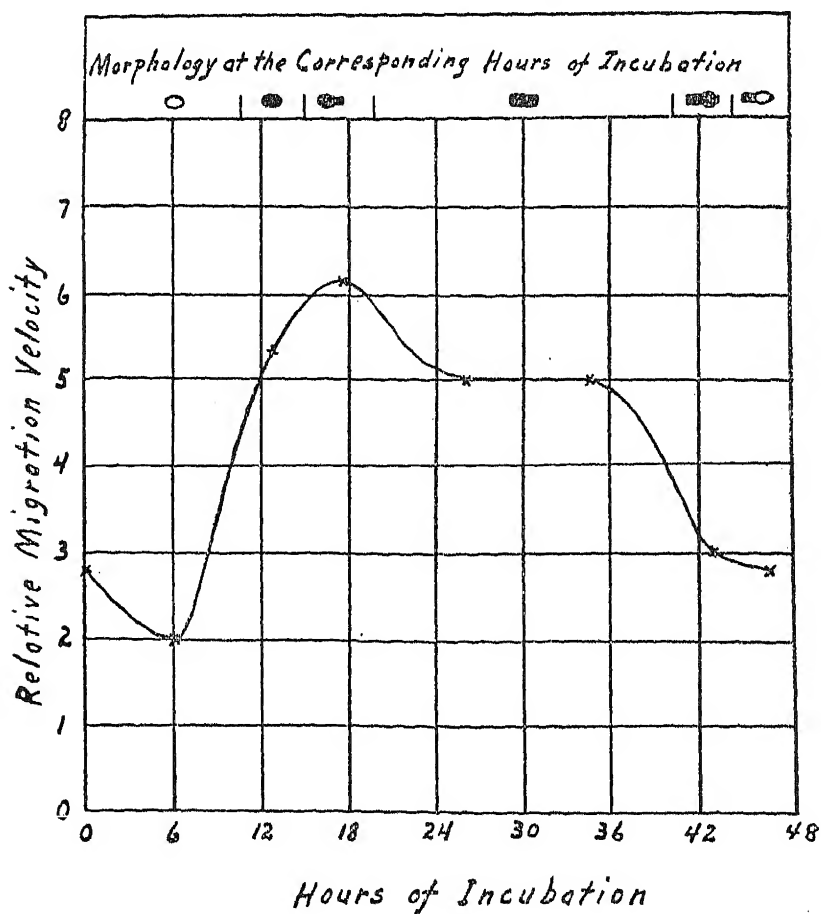


FIG. 4. AVERAGE CATAPHORETIC POTENTIAL OF *CL. BOTULINUM* AFTER VARIOUS PERIODS OF INCUBATION

seen to decrease at first and then increase to a maximum, followed by a decrease to about that of the original spores. Whether the change in the cataphoretic potential indicates an actual change in

the nature of the cell proteins, the accumulation of non-diffusible by-products, or some other factor is impossible to say from the data at hand.

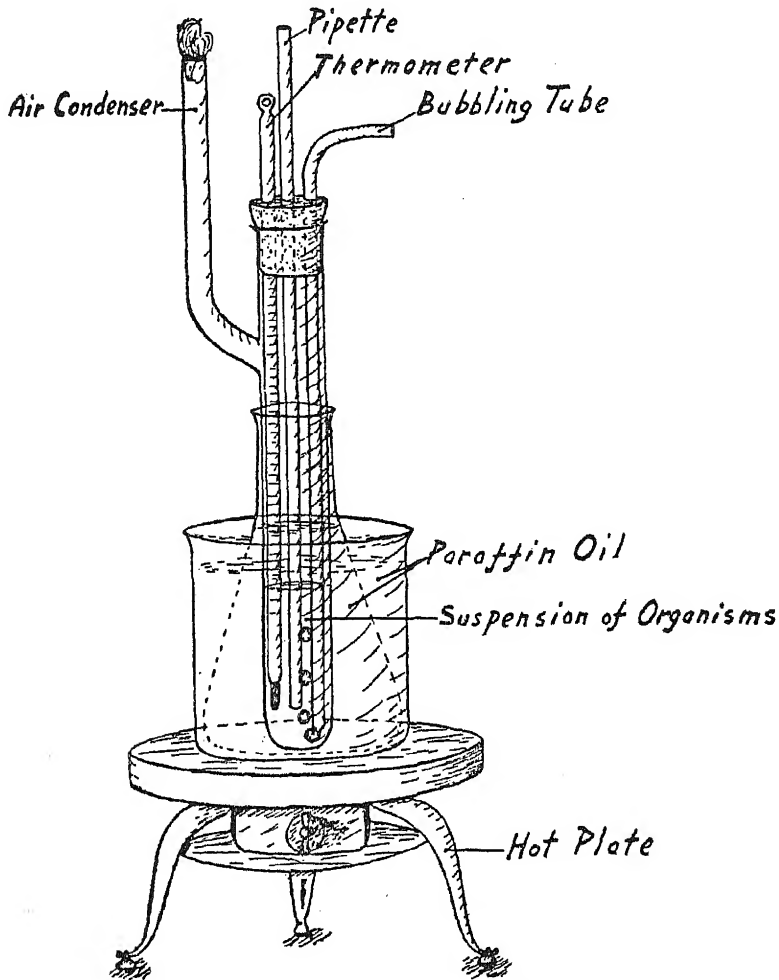


FIG. 5. APPARATUS FOR DETERMINING THE THERMAL DEATH TIME-TEMPERATURE OF *CL. BOTULINUM* AT VARIOUS PERIODS OF THE SPORE CYCLE

Changes of thermal death time-temperature

A determination of the thermal sensitivity of an organism in the process of germination or sporulation can obviously not be made in the ordinary way. The method adopted was to raise the temperature of the suspension of organisms gradually, and remove samples to agar shakes at 30°, 60°, 70°, 80°, and 90°C. respectively. The time was also taken. The apparatus consisted of a test tube fitted with an air condenser, and a three-hole rubber stopper. Through the holes in the stopper was put a thermometer, a capillary pipette for withdrawing samples, and a tube from a nitrogen tank. Oxygen-free nitrogen was kept bubbling through the suspension continuously to keep air out, and stir the suspension. A drawing of the apparatus is shown in figure 5. The time for the temperature to rise from about 25° to 60°C. was about twenty minutes, from 60° to 70°C. about six minutes, from 70° to 80°C. about seven minutes, and from 80° to 90°C. about eight minutes. The data show a gradual increase of thermal sensitivity as the organism germinates, and a decrease as it sporulates. It is interesting to note the low resistance of a majority of the original spores, only ten per cent surviving up to 90°C. The average thermal death point of most of these spores would then be between 80° and 90°C. The newly formed spores were even less resistant; only 1 to 2 per cent survived up to 90°C., 10 per cent up to 80°C., 20 per cent up to 70°C. and 50 per cent up to 60°C. The preliminary heating of the original spores to 70°C. for ten minutes accounts for the difference in heat resistance between these spores and those produced later. The observation is general that only a fraction of the spores formed in any culture are capable of germinating. This is especially true if the spores have been subjected to unfavorable conditions of any kind. Most spores are, therefore, probably failures as far as their chief function (?) is concerned of tiding the race of organisms over periods of unfavorable environmental conditions. It is doubtful if other media would have produced more heat resistant spores. An average of the different readings is plotted in figure 6.

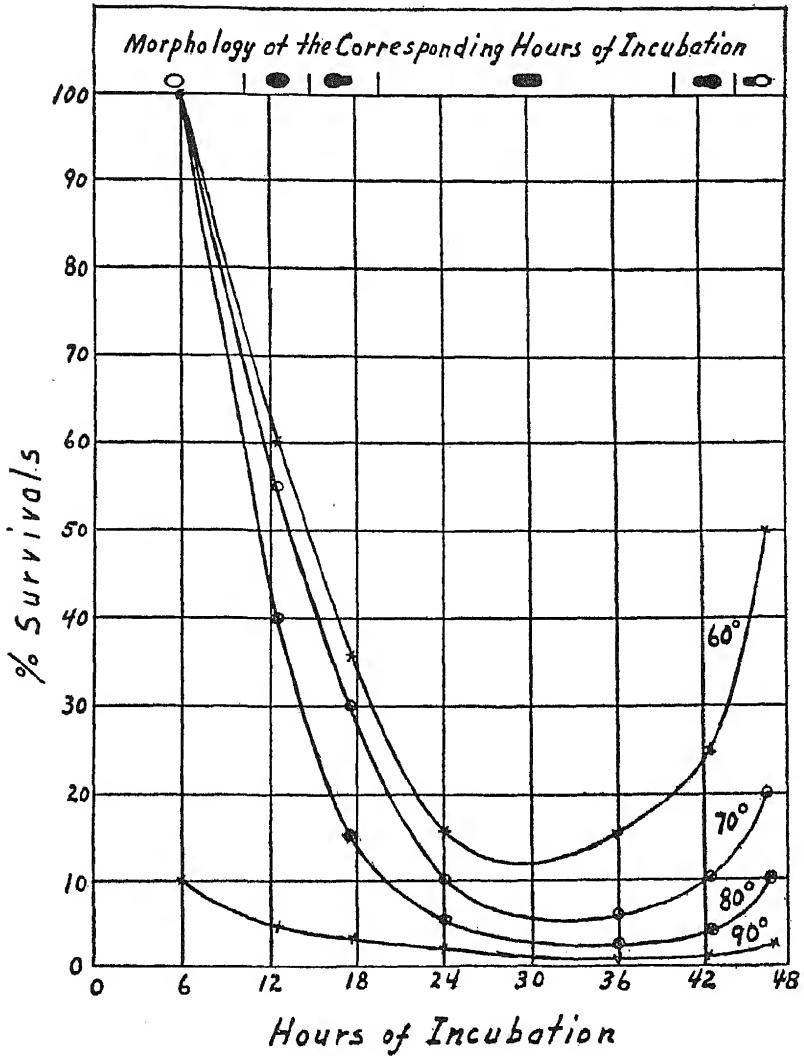


FIG. 6. AVERAGE PER CENT OF *CL. BOTULINUM* IN VARIOUS GROWTH PHASES SURVIVING HEATING AT VARIOUS TEMPERATURES

Change of oxygen sensitivity

Some anaerobes in the vegetative state are quickly killed by exposure to air. Previous work on the subject gives results which differ considerably. The present experiment was undertaken to correlate the change in sensitivity of *Cl. botulinum* with change in morphology as it passed through the spore cycle. The method was to grow the organisms in bouillon and at different morphologic stages remove a tube from the anaerobic jar and streak a loopful of the culture over an agar slant. The agar slants were then left exposed to the air, upside down, for different lengths of time; they were incubated anaerobically, and the number of colonies which developed counted. In the first few experiments agar slants of about pH 6, pH 7, and pH 8 were used, and exposed at temperatures of 8° and 37°C. (ice box and incubator). In this manner the effect on sensitivity of both pH and temperature could be obtained simultaneously. No differences in sensitivity of the organisms exposed at the different H-ion concentrations were found, nor in those exposed at the different temperatures. The shortest exposure in most of these cases was five minutes and it is possible that if shorter exposures had been tried a differential effect might have been found. In the later experiments only one kind of agar slants was used, pH 6.8 to 7, and all were exposed at 37°C. To express the results concisely is most difficult. In only one case of a considerable number was there obtained what may be considered a differential effect of different lengths of exposure. In this case the organisms were close to, if not in, the beginning stages of sporulation. The results of this one case were as follows:

Length of exposure minutes	Survivals per cent
1	100
6	100
11	25
46	15
70	5

These results may have been accidental. The method of expressing the results in figure 7 was to estimate the number of survivals after exposure to air for one hundred minutes and for five minutes.

It seems that organisms which are at all sensitive are generally killed by exposure of two minutes or less (two minutes was the shortest time possible with the method used). It appears from

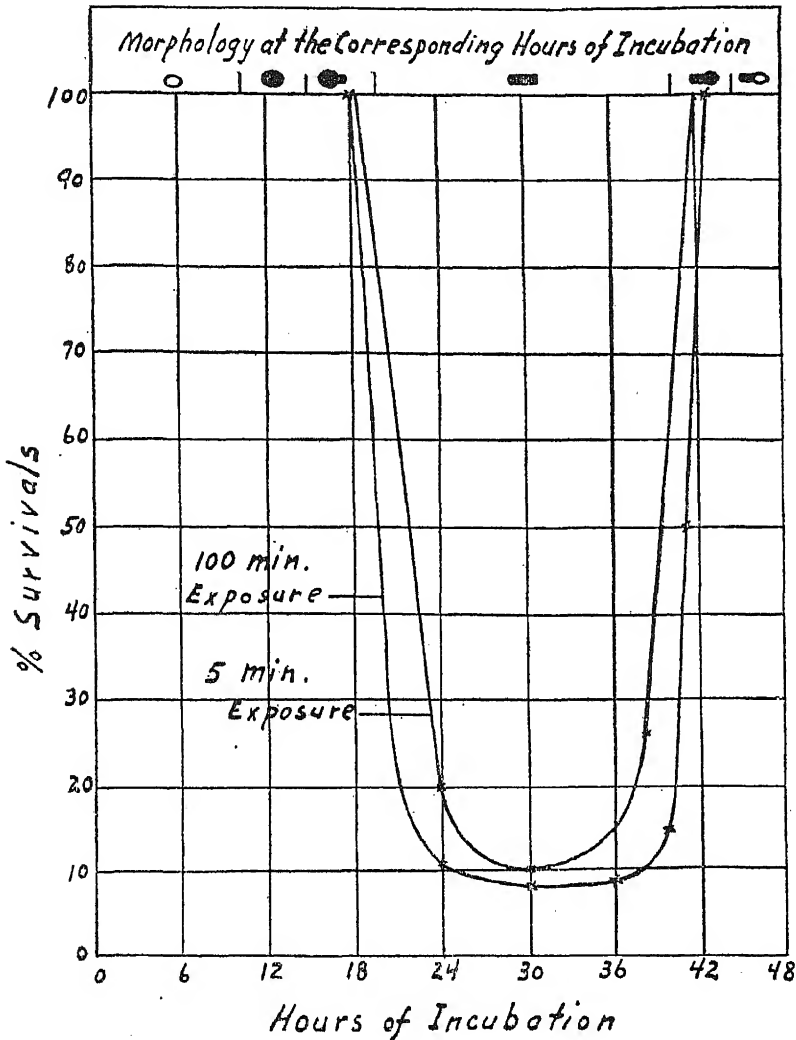


FIG. 7. AVERAGE PER CENT OF *CL. BOTULINUM* IN VARIOUS GROWTH PHASES SURVIVING EXPOSURE TO OXYGEN FOR FIVE AND ONE HUNDRED MINUTES

the curve that only vegetative cells are oxygen sensitive. Neither germinating spores nor sporulating vegetative cells show any appreciable sensitivity. The fact that the curves do not in any case reach the 0 line must not be taken to indicate that some vegetative cells are insensitive to oxygen but rather that there are present a number of ungerminated spores, which later germinate. It is possible that vegetative cells which do not sporulate lose their sensitivity as they become older. No data were obtained on this score.

Toxin production

The usual procedure in the production of botulinum toxin has been to cultivate the organisms for about a week. In a culture where at least 90 per cent of the organisms sporulate within forty-eight hours, interesting light on toxin production could perhaps be obtained. White mice were injected intraperitoneally with different quantities of supernatant broth after centrifuging out the organisms. In each case the total amount injected was $\frac{1}{2}$ cc. Antitoxin was injected to control the highest concentration injected. Every injection was made in duplicate. The three sets of data check very closely, and the average of the three is plotted in figure 8.

It is interesting to note that very little toxin is produced until the organism has reached the height of its growth stage. As sporulation begins, the toxin titer falls rather quickly and it seems as if no further toxin is produced. Upon exposure to air the toxin is destroyed very quickly. An experiment was made by mixing four tubes of toxic bouillon, and dividing the mixture into four tubes, care being taken to avoid exposure to air as much as possible. Two of these tubes were placed immediately under anaerobic conditions. The other two were thoroughly shaken in the air from time to time. The toxicity of the pooled broth was between 10,000 and 100,000 mice M.L.D. per cubic centimeter. After twenty-one hours the toxin titers were again tested. The anaerobic tubes gave a titer the same as the original. The exposed tube gave a titer of slightly over 100 M.L.D. After five

days the titer of the anaerobic tubes was somewhat over 100 M.L.D., and the aerobic tube somewhat over 10 M.L.D.

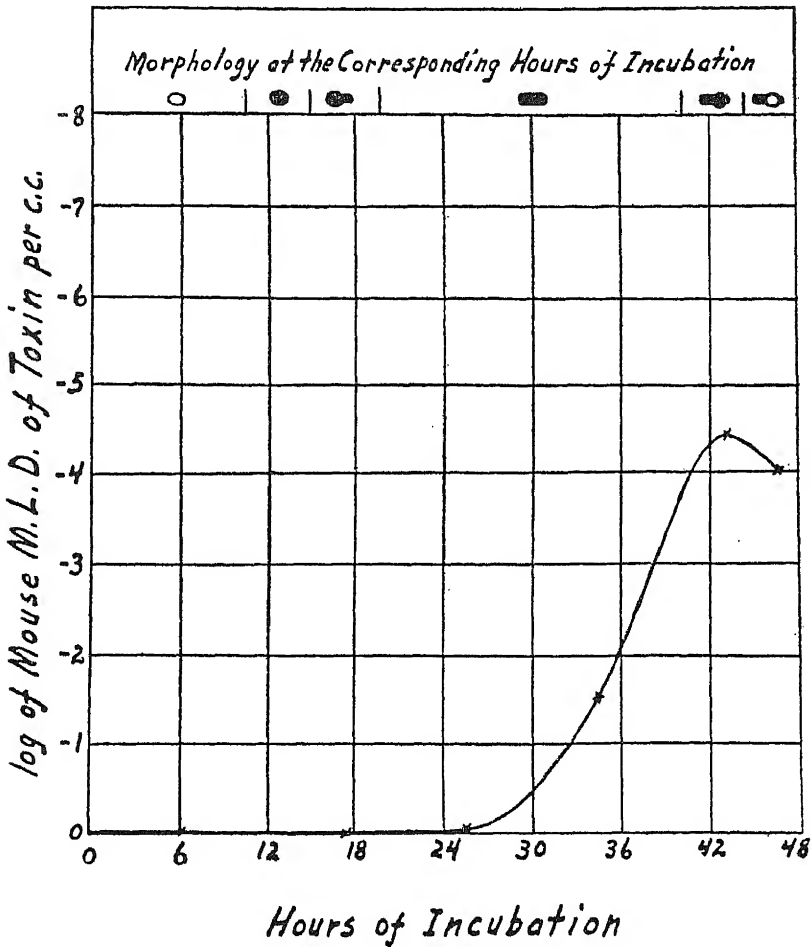


FIG. 8. AVERAGE TOXICITY OF THE *CL. BOTULINUM* CULTURES AT VARIOUS GROWTH PHASES

SUMMARY

With *Cl. botulinum*, and to a less definite degree with other organisms, the optimum hydrogen-ion concentration for sporula-

tion in a phosphate buffered medium is slightly acid and in a number of cases found to be at a pH of about 6.2 to 6.3.

Fermentable carbohydrates have an inhibiting effect on sporulation, mainly due to the production of acid and consequent high hydrogen-ion concentration produced. Whenever sufficient acid is produced to bring the pH below that at which growth can occur no sporulation takes place. With *Cl. botulinum* the limiting hydrogen-ion concentration for growth is about pH 6.

Increasing concentrations of inorganic salts will inhibit sporulation before inhibiting growth. In the case of *Cl. botulinum* the chlorides seem to be quite effective in inhibiting sporulation. Nitrates in concentration of 1 per cent have an inhibiting effect on the sporulation of *Cl. botulinum*. Ammonium salts have a stimulating effect on the sporulation of *Cl. botulinum*, as have also sulphates but to a lesser extent.

Certain elements seem to be more essential for sporulation than for growth. *Cl. botulinum* does not sporulate in a medium consisting of peptone only. Addition of various salts to peptone shows that ammonium and phosphate ions will induce sporulation while other ions will not. The calcium ion seems to have an added stimulating effect when added to ammonium and phosphate ions.

Increasing concentrations of oxygen inhibit sporulation before inhibiting growth. The optimum oxygen concentration for the sporulation of the anaerobes seems to be as close to 0 as it is possible to get by ordinary means. Neither growth nor sporulation was obtained with a number of anaerobes at an oxygen concentration of 2 cm. of mercury. Except with *Cl. tetani* and *Cl. novyi* some sporulation occurred at an oxygen tension of 1 cm. of mercury. In the case of the aerobes no sporulation was obtained at an oxygen tension of 2 cm. of mercury, although fair growth was observed.

A number of experiments were performed with *Cl. botulinum* to study the change of various properties as the organisms went through the spore cycle. The properties studied were: Morphology, cataphoretic potential, thermal resistance, oxygen sensitivity, pH of medium, and toxicity of medium. In each case the

change of the different properties of the organisms was correlated with the morphology.

The cataphoretic potential of spores was lower than that of the specific vegetative cells. The germinating spores had the highest potential.

Thermal death point determinations showed that a great number of the spores produced were not very resistant to high temperatures and must be regarded as failures. Only 1 to 2 per cent of the newly formed spores survived heating at 90°C. for a few minutes, 10 per cent survived 80°C., 20 per cent survived 70°C., and 50 per cent survived 60°C.

The experiment on oxygen sensitivity showed that only vegetative cells were oxygen sensitive. These were so sensitive that most of them were killed by less than two to three minutes exposure to air. The change from the nonsensitive spore to the exceedingly sensitive vegetative cell was so abrupt that only in a few cases was the sensitivity of the intermediary stages obtained.

Toxin production did not begin appreciably until the organisms were about to stop multiplying and sporulation began. During the period of sporulation the toxin titer increased very rapidly and reached a maximum at the time sporulation was complete. It then decreased due to the destruction of the toxin, especially if the toxin was allowed to come in contact with the air.

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DEVELOPMENT OF FLAGELLA ON GERMINATING SPORES¹

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Received for publication December 3, 1930

The only observation on the development of flagella on germinating spores which has come to my attention is that of Klein (1889). Klein used an organism he called *B. leptosporus* n.sp. (Klein). He made hanging drop preparations of the spores and observed them continuously at a temperature of about 35°C. The germination was generally complete in five and three-quarters hours. No motion of the organisms during germination was observed. Not until six hours and fifty minutes did characteristic motion begin to appear. At this time many four-membered chains of cells could be seen. From this observation it is impossible to tell exactly when and how flagella develop on the newly formed cell.





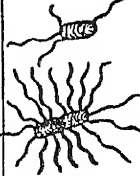

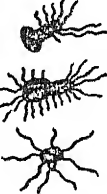

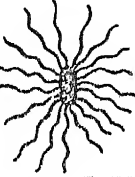


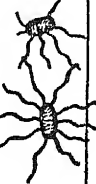

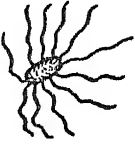
Working with a number of motile spore-forming bacteria including *B. vulgatus*, *B. cereus*, and *B. flavus* the author set about making flagella stains of the germinating spores. The stain used was that described by Leifson (1930). The method was essentially as follows: Week-old agar slant cultures, containing practically no vegetative cells, were suspended in distilled water and the suspension heated to 70°C. for ten minutes to kill any vegetative cells. A number of bouillon tubes were then heavily inoculated by means of a pipette with the heated suspension. At different intervals of time a tube was removed from the incu-

¹ Part of a dissertation submitted to the Department of Hygiene and Bacteriology of the University of Chicago to fulfil certain requirements for the degree of Doctor of Philosophy.

bator, a hanging drop made to determine motility and the organisms washed in distilled water. Flagella stains were made of

Table 1
Time and Manner of Development of Flagella
on Newly Formed Vegetative Cells.

Motility and Morphology after Indicated Hours of Incubation

Organisms used	1 $\frac{1}{4}$ Hrs.	1 $\frac{1}{2}$ Hrs.	1 $\frac{3}{4}$ Hrs.	2 Hrs.	2 $\frac{1}{2}$ Hrs.
<u>B. vulgatus</u>					
Motility	no motile forms	no motile forms	no motile forms	some motile forms	many motile forms
Morphology					
<u>B. cereus</u>					
Motility	no motile forms	some motile forms	-	sluggish motility	active motility
Morphology					
<u>B. flavus</u>					
Motility	no motile forms	some motile forms	many motile forms	good motility	good motility
Morphology					

these washed organisms. The results with three of the organisms are given in table 1.

It is evident from table 1 that flagella begin to grow out of the organisms while they are still in the process of germination. The flagella do not grow out all at once but one by one. They evidently grow very rapidly; perhaps as much as 1 micron every two to three minutes. The organisms evidently become motile with only 2 or 3 short flagella present.

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STUDIES ON CARBON METABOLISM OF ORGANISMS OF THE GENUS MYCOBACTERIUM

II. UTILIZATION OF ORGANIC COMPOUNDS IN A SYNTHETIC MEDIUM

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Received for publication January 2, 1931

INTRODUCTION

It was emphasized by the author in a previous report (1930) that the demonstration of carbohydrates utilized by organisms of the genus *Mycobacterium* presents a somewhat different problem from that involved in demonstrating carbohydrate utilization by most other bacteria. Whereas it has generally been customary to demonstrate carbohydrate utilization by noting the accumulation of acid in the media it was shown that this method is not applicable in the case of organisms of the genus *Mycobacterium*. Similarly, it was emphasized in the previous report that determinations of the reaction curves, using standard meat infusion broth as the basis of the media, are of little if any value in determining whether carbohydrates are utilized.

When direct quantitative determinations were made it was found that a number of carbohydrates were utilized by these organisms. A study was also made of the method of utilization and the evidence obtained indicated that no intermediate products of carbohydrate cleavage accumulate in the media during the growth of organisms of this genus.

The method employed for determining utilization gave information concerning utilization of the reducing sugars only. In order to determine the extent to which other carbon compounds

were utilized another method of study had to be devised. Briefly stated, a synthetic medium was used which contained the carbon compound under investigation as the sole carbon source. The appearance of growth and reaction change in the media indicated utilization. The details of the method will be given below.

That bacteria will grow in media of relatively simple composition has been known for a long time. The earlier work in which synthetic media were used in studies of the metabolism of the tubercle bacillus has been reviewed by Wherry (1913). A more extensive review and discussion was published later by Long (1923).

Various synthetic media were used by Wherry (1913) in a study of the acid fastness and non-acid fastness of organisms in cultures of a saprophytic strain of the tubercle bacillus. In one of these media ammonium chloride was used as the sole source of nitrogen.

Kendall et al. (1914) used a medium containing ammonia as the sole source of nitrogen and obtained growth of several species of acid-fast organisms. In addition their medium contained phosphate, sulphate, and sodium chloride, and had glycerol as the carbon source. They were able to obtain growth of a rapidly growing strain and slight growth of a more virulent strain of *Myc. tuberculosis* in this medium.

Later H. Braun and Cohn-Bronner (1921) adopted a similar method for the study first of *Bacillus para-typhosus* Beta and later (1921a) (1921b) extended their work to include *B. para-typhosus* Alpha and *B. typhosus*. S. Kondo and associates (1924) (1924a) (1924b) (1925) working in the same laboratory carried the work further in a study of several species of the genus *Mycobacterium*. An extensive study was made of the elemental and energy requirements of the latter organisms. A simple medium of known chemical composition that would permit growth was found. Then, by varying the ingredients the authors were able to determine the substances essential to growth; for example, the nitrogen source was varied keeping other factors constant, then the carbon source varied using a nitrogen source that was known to be utilizable, and finally the various mineral elements were

varied while keeping known utilizable nitrogen and carbon sources constant. It was found by these workers that ammonia in the form of ammonium sulphate sufficed as a nitrogen source in the presence of utilizable carbon. Only organic carbon compounds, however, would supply the necessary energy for growth. Growth appeared, then, only when a carbon compound that could be utilized by the organisms was contained in the medium.

The latter principle seemed to offer a simple, yet effective, means of determining the extent to which carbohydrates and derived carbon compounds are utilized by the Mycobacteria. The investigation reported below is therefore an adaptation and extension of the work of Kondo and his associates. Certain changes in the methods which tend to simplify and make for greater definiteness have been made.

METHODS

The medium used for these determinations contained the following ingredients dissolved in distilled water to make the percentage concentration indicated:

	<i>per cent</i>
NaCl.....	0.5
(NH ₄) ₂ SO ₄	0.5
MgSO ₄	0.005
KH ₂ PO ₄	0.04
K ₂ HPO ₄	0.16
Carbon source.....	0.5
Phenol red.....	0.001

The various reagents, except the carbon source and the phenol red, were weighed out into a volumetric flask. One cubic centimeter of stock 0.1 per cent solution phenol red for each 100 cc. of final medium was added, and then the flask was filled to the mark with distilled water. This constituted the stock medium and could be kept indefinitely in the unsterilized form without significant bacterial multiplication occurring. It is essential for this work that the phenol red be made up in a sodium hydroxide solution as recommended by Clark (1920) and not as an alcoholic solution. If the latter be added an abundant bacterial growth will occur in the unsterilized stock medium at room temperature.

Just prior to sterilization the carbon source (glucose, fructose, or sodium lactate, etc., as desired) was weighed out, or if liquid measured with a pipette, added to a graduate cylinder and the above stock medium added in such an amount as would give a 0.5 per cent concentration of the carbon source.

Immediately after solution had been effected the respective media were sterilized by filtration. Subsequent tubing was accomplished as outlined in the reaction curve determinations in a previous report (1930). Incubation at 37°C. for a period of one week followed as a check on the sterility of the media. Contaminants were seldom encountered but when present they became evident within two or three days as demonstrated by turbidity of the medium associated with reaction change.

The following carbohydrates and derived carbon compounds were tested for utilization by each organism: monosaccharides—glucose, arabinose, fructose, and galactose; disaccharides—maltose, lactose, and sucrose; polysaccharides—inulin and raffinose; alcohols—mannitol, glycerol, ethyl alcohol, and salicin; salts of organic acids—sodium lactate, sodium citrate, and sodium acetate.

The organisms used in this work are the same as those reported in a former article (1930). The species names of these organisms are given in the tables.

Inoculations were made into the synthetic media from glycerol agar cultures. Whenever possible, a thin section of inoculum was made to float on the surface of the media. Where this could not be done, as with those organisms which produced a butyrous or mucoid growth, the organisms were smeared on the side of the tube at the surface of the liquid.

The cultures of the various organisms were kept at temperatures which permitted optimum growth. The cultures of *Myco. avium* were incubated at 41°C. while the cultures of *Myco. chelonae*, *Myco. ranae*, and *Myco. butyricum* were incubated at 20°C. The remaining organisms were incubated at 37°C. The air of the incubator was maintained at a high relative humidity throughout the incubation in order to minimize evaporation. The duration of incubation varied as shown in tables 1, 2, and 3.

TABLE 1*
Changes in pH and growth obtained in synthetic media which contained as the sole carbon source the carbon compounds indicated

	GLUCOSE			FRUCTOSE			GALACTOSE			ARABINOSE			MANNITOL		
	pH 14 days	pH 28 days	Growth	pH 14 days	pH 28 days	Growth	pH 14 days	pH 28 days	Growth	pH 14 days	pH 28 days	Growth	pH 14 days	pH 28 days	Growth
Control.....	7.0	6.8	—	7.0	6.8	—	7.0	6.8	—	7.0	6.8	—	7.0	6.8	—
<i>Myc. tuberculosis</i> H-37†.....	6.8	6.6	—	6.8	6.6	—	6.8	6.6	—	6.8	6.6	—	6.8	6.6	—
<i>Myc. tuberculosis</i> M-1†.....	6.8	6.6	—	6.8	6.6	—	6.8	6.6	—	6.8	6.6	—	6.8	6.6	—
<i>Myc. tuberculosis</i> (saprophytic).....	±6.0±6.0	±6.0	++	±6.0±6.0	±6.0	++	±6.0±6.0	±6.0	++	±6.0±6.0	±6.0	++	±6.0±6.0	±6.0	++
<i>Myc. tuberculosis</i> (bovis)†.....	6.8	±6.0	+	6.8	6.6	—	6.8	6.6	—	6.8	6.6	—	6.8	6.6	—
<i>Myc. leprae</i> (282).....	7.0	6.8	—	6.6±6.0	±6.0	+	7.0	6.8	—	7.0	6.8	—	7.0	6.8	—
<i>Myc. avium</i>	7.0	6.8	—	7.0	6.8	—	7.0	6.8	—	7.0	6.8	—	7.0	6.8	—
<i>Myc. ranarum</i>	6.8	6.2	+	±6.0±6.0	±6.0	+	7.0	6.6	—	7.0	6.4	—	6.8±6.0	6.8	—
<i>Myc. chelonae</i> †.....	7.0	±6.0	+	7.0	6.8	—	7.0	6.8	—	7.0	6.8	—	7.0	6.8	—
<i>Myc. smegmatis</i>	±6.0±6.0	±6.0	++	±6.0±6.0	±6.0	++	±6.0±6.0	±6.0	++	±6.0±6.0	±6.0	++	±6.0±6.0	±6.0	++
<i>Myc. butyricum</i> †.....	6.8	6.6	+	6.4±6.0	±6.0	++	7.0	6.6	+	6.6	6.4	+	7.0±6.0	±6.0	+
<i>Myc. berolinensis</i>	±6.0±6.0	±6.0	++	±6.0±6.0	±6.0	++	±6.0±6.0	±6.0	++	±6.0±6.0	±6.0	++	±6.0±6.0	±6.0	++
<i>Myc. friburgensis</i>	±6.0±6.0	±6.0	++	±6.0±6.0	±6.0	++	7.0	6.6	±	7.0	6.8	—	7.0	6.8	—
<i>Myc. stercois</i>	±6.0±6.0	±6.0	++	±6.0±6.0	±6.0	++	±6.0±6.0	±6.0	+	±6.0±6.0	±6.0	++	6.4±6.0	±6.0	+
<i>Myc. phlei</i>	±6.0±6.0	±6.0	++	±6.0±6.0	±6.0	++	6.6±6.0	±6.0	++	±6.0±6.0	±6.0	++	±6.0±6.0	±6.0	++

Explanation of symbols: ± 6.0 indicates that the pH is less than 6.4; — indicates no growth in any determination; ± indicates slight growth in some determinations but not in every case; + indicates slight to moderate growth in first culture and growth in subcultures; ++ indicates moderate to abundant growth in first culture and also in subcultures.

* Initial reaction of all media was pH 7.2.

† Results shown are after 28 and 75 days incubation instead of 14 and 28 days.

‡ Results are after 14 and 90 days incubation instead of 14 and 28 days.

TABLE 2

Changes in pH and growth obtained in synthetic media which contained as the sole carbon source the carbon compounds indicated

	GLYCEROL*			ETHYL ALCOHOL†			LACTIC ACID‡			CITRIC ACID*			ACETIC ACID††		
	pH 14 days	pH 28 days	Growth	pH 14 days	pH 28 days	Growth	pH 14 days	pH 28 days	Growth	pH 14 days	pH 28 days	Growth	pH 14 days	pH 28 days	Growth
Control.....	6.8	6.8	—	7.0	7.0	—	7.0	6.8	—	7.0	6.8	—	7.0	6.8	—
<i>Myc. tuberculosis</i> H-37††	6.6	6.4	±	7.0	7.0	—	7.0	6.8	—	6.8	6.8	—	6.8	6.8	—
<i>Myc. tuberculosis</i> M-1††	6.4	±6.0	+	7.0	7.0	—	7.0	6.8	—	6.8	6.8	—	6.8	6.8	—
<i>Myc. tuberculosis</i> (saprophytic).....	±6.0	±6.0	+	7.0	±6.0	+	±8.6	7.4	+	6.9	7.3	+	8.6	7.4	+
<i>Myc. tuberculosis</i> (bovis)††	6.6	±6.0	+	7.0	7.0	—	6.8	6.8	—	6.8	6.8	—	6.8	6.6	±
<i>Myc. leprae</i> (282).....	6.2	±6.0	+	6.8	±6.0	+	7.0	7.0	±	6.9	6.8	—	8.6	7.4	±
<i>Myc. avium</i>	6.8	6.4	+	7.0	7.0	—	7.0	6.8	—	6.9	6.8	—	7.0	7.2	±
<i>Myc. ranae</i>	6.6	±6.0	+	±6.0	±6.0	+	8.6	7.6	+	6.9	6.8	—	8.0	8.4	±
<i>Myc. chelonae</i>	6.8	6.2	+	7.0	7.0	—	7.0	6.8	±	7.6	8.2	+	8.6	8.6	+
<i>Myc. smegmatis</i>	±6.0	±6.0	+	±6.0	±6.0	+	±8.6	7.2	+	7.2	7.8	+	8.6	7.8	+
<i>Myc. butyricum</i>	6.2	±6.0	+	7.0	7.0	±	7.0	7.0	±	6.9	6.8	±†	7.3	7.3	±
<i>Myc. berolinensis</i>	±6.0	±6.0	+	±6.0	±6.0	+	±8.6	7.2	+	6.9	7.6	+	8.6	8.4	+
<i>Myc. friburgensis</i>	±6.0	±6.0	+	±6.0	±6.0	+	±8.6	7.2	+	6.9	6.8	±**	8.6	7.8	+
<i>Myc. stercois</i>	±6.0	±6.0	+	±6.0	±6.0	+	±8.6	7.2	+	6.9	7.3	+	8.6	7.8	+
<i>Myc. phlei</i>	±6.0	±6.0	+	±6.0	±6.0	+	±8.6	7.2	+	6.9	7.4	+	8.6	7.8	+

Explanation of symbols: ± 8.6 indicates that pH is greater than 8.4; other symbols as in table 1.

* Initial pH was 7.0.

† Incubation 90 days, very slight growth obtained.

‡ Initial pH was 7.2.

§ Initial pH was 7.6.

¶ After 90 days incubation there was definite growth with pH of 8.0.

** Growth after 75 days.

†† Initial pH was 7.3.

‡‡ Results shown are after 28 and 75 days instead of 14 and 28 days incubation.

TABLE 3

Changes in pH and growth obtained in synthetic media which contained as the sole carbon source the carbon compounds indicated

	MALTOSE*			SUCROSE†			LACTOSE*			RAFFINOSE‡			INULIN*			SALICIN		
	pH 14 days	pH 28 days	Growth	pH 14 days	pH 28 days	Growth	pH 14 days	pH 28 days	Growth	pH 14 days	pH 28 days	Growth	pH 14 days	pH 28 days	Growth	pH 14 days	pH 28 days	Growth
Control.....	6.9	6.6	—	7.1	6.8	—	7.0	6.6	—	7.0	6.7	—	7.2	7.2	—	7.2	7.0	—
<i>Myc. tuberculosis</i> H-37§.....	6.9	6.6	—	6.8	6.8	—	6.6	6.6	—	6.7	6.6	—	7.2	7.2	—	7.0	6.8	—
<i>Myc. tuberculosis</i> M-1§.....	6.9	6.6	—	6.8	6.8	—	6.6	6.6	—	6.7	6.6	—	7.2	7.2	—	7.0	6.8	—
<i>Myc. tuberculosis</i> (saprophytic).....	6.9	6.6	—	7.1	6.8	—	7.0	6.6	—	7.0	6.7	—	7.2	7.2	—	7.2	7.0	—
<i>Myc. tuberculosis</i> (<i>bovis</i>)§.....	6.9	6.6	—	6.8	6.8	—	6.6	6.6	—	6.7	6.6	—	7.2	7.0	—	7.0	6.8	—
<i>Myc. leprae</i> (282).....	6.9	6.6	—	±6.0	±6.0	+	7.0	6.6	—	7.0	6.7	—	7.2	7.2	—	7.2	7.0	—
<i>Myc. avium</i>	6.9	6.6	—	7.1	6.8	—	7.0	6.6	—	7.0	6.7	—	7.2	7.2	—	7.2	7.0	—
<i>Myc. ranae</i>	6.9	6.6	—	7.1	6.8	—	7.0	6.6	—	7.0	6.7	—	7.2	7.2	—	7.2	7.0	—
<i>Myc. chelonae</i>	6.9	6.6	—	7.1	6.8	—	7.0	6.6	—	7.0	6.7	—	7.2	7.2	—	7.2	7.0	—
<i>Myc. smegmatis</i>	6.9	6.6	—	7.1	6.8	—	7.0	6.6	—	7.0	6.7	—	7.2	7.2	—	7.2	7.0	—
<i>Myc. butyricum</i>	6.9	6.6	—	7.1	6.8	—	7.0	6.6	—	7.0	6.7	—	7.2	7.2	—	7.2	7.0	—
<i>Myc. bercliniensis</i>	±6.0	±6.0	+	7.1	6.8	—	7.0	6.6	—	7.0	6.7	—	7.2	6.6	±¶	6.8	6.0	±¶
<i>Myc. friburgensis</i>	6.9	6.6	—	7.1	6.8	±	7.0	6.6	—	7.0	6.7	—	7.2	7.2	—	7.2	7.0	—
<i>Myc. stercusis</i>	6.9	6.6	±	7.1	6.8	—	7.0	6.6	—	7.0	6.7	—	7.2	6.6	±¶	6.8	6.0	±¶
<i>Myc. phlei</i>	6.9	6.6	—	7.1	6.8	—	7.0	6.6	—	7.0	6.7	—	7.2	7.2	—	7.2	7.0	—

Explanation of symbols: see table 1.

* Initial pH was 7.2.

† Initial pH was 7.4.

‡ Initial pH was 7.0.

§ Results shown are after 28 and 75 days instead of 14 and 28 days incubation.

¶ Results shown are after 45 days incubation.

All tests were run in duplicate. In the first series of determinations, in which ten different organisms were used, subculture into a tube of fresh media containing the same carbohydrate was made from each test culture in which there was any evidence of utilization (*viz.*, growth). In addition a new set-up, again in duplicate, was made from the stock glycerol agar culture. Where demonstrable utilization occurred in all the six tests (that is, three series of duplicate tests) it was considered evidence that the carbohydrate under investigation was definitely utilized. The results in the six test cultures were almost uniformly parallel. In the few cases in which the results were not entirely definite subsequent tests were made to ascertain whether or not utilization occurred. With the remaining organisms reported, at least two and in some cases more than two determinations in duplicate were made in each case.

As stated above the results were read on a basis of two criteria. The first of these was whether or not observable growth occurred. That growth depended upon utilization of the carbohydrate is shown by the fact that with several of the carbon compounds used no growth was obtained. At the termination of incubation each culture in which there was any evidence of growth was checked by staining, using Ziehl-Neelson stain, in order to rule out growth of contaminants.

As a second criterion for utilization, the reaction changes of the cultures were followed. This has not been done by previous workers who employed this method for determining carbohydrate utilization. The type of reaction changes differed, as will be shown later, depending upon the carbon compound included in the media. The change in hydrogen ion concentration in each set of determinations was controlled by uninoculated tubes of media subjected to the same incubation conditions. This was essential because there was some change in reaction in the control tubes as well as in the inoculated tubes as incubation proceeded.

The hydrogen ion concentrations were determined colorimetrically, the series of standards being prepared as outlined in a previous article (1930). These standards were graded on a basis of a difference in pH of 0.2 from pH 5.8 to 8.6. Since phenol red was

used as indicator, readings below pH 6.6 or above pH 8.2 were only approximations. Nevertheless, these readings were sufficiently accurate for the purpose intended, since, in the final analysis, it was the difference between the inoculated cultures and the uninoculated controls that was of importance.

In recording the results, any reactions noted which were below pH 6.4 or above pH 8.4 as shown by comparison with the standards are recorded as plus or minus (\pm) 6.0 or 8.6 respectively.

In tabulating growth obtained the following symbols are employed: minus ($-$) indicates no growth in any determinations; plus or minus (\pm) indicates slight growth in some of the determinations but not in every case; one plus ($+$) indicates light to moderate growth in the first cultures and growth in sub-cultures; two plus ($++$) indicates moderate to abundant growth in both the first culture and the subcultures.

RESULTS

The results are tabulated in tables 1, 2, and 3. These results represent a composite of the findings,—in most cases of three or more duplicate determinations. In the case of the slow growing strains of *Myco. tuberculosis (hominis)* and (*bovis*) when no evidence of utilization was shown after extended periods of incubation in either of the first two cultures the determinations were not repeated.

In still other cases the incubation period had to be prolonged before utilization could be demonstrated. For example it was necessary to incubate some of the cultures of *Myco. ranae*, *Myco. chelonae*, and *Myco. butyricum* for as long as ninety days before conclusive results could be obtained. All such exceptions as the two given are noted in the tables.

The tables show the hydrogen ion concentration of the cultures at the end of fourteen and twenty-eight days of incubation and the growth at the end of twenty-eight days (except in the cases which are marked otherwise). It should be noted that the general tendency was for the changes in hydrogen ion concentration to be proportional to the growth. In those cases in which there was a rapid change in pH there was almost constantly a rela-

tively abundant growth. On the other hand where the growth was slight a reaction change beyond that found in the uninoculated controls was in some cases hardly discernible. These points are emphasized by the results in the tables.

The results of the utilization determinations are summarized in table 4. It is to be noted that glycerol was utilized by all

TABLE 4
Summary of results on utilization determinations

	MYCO. TUBERCULOSIS H-37	MYCO. TUBERCULOSIS M-1	MYCO. TUBERCULOSIS SAPROPHYTE	MYCO. TUBERCULOSIS BOVIS	MYCO. LEPRAE 282	MYCO. AYIUM	MYCO. RANAE	MYCO. CHELONEI	MYCO. SMEGMATIS	MYCO. BUTYRICUM	MYCO. BEHOLINENSIS	MYCO. FRIGIDIGENSIS	MYCO. STERCUS	MYCO. PHLEI
Glucose.....	±	0	+	+	±	0	+	+	+	+	+	+	+	+
Fructose.....	0	0	+	0	±	0	+	0	+	+	+	+	+	+
Galactose.....	0	0	+	0	0	0	±	0	+	±	+	±	+	+
Arabinose.....	0	0	+	0	0	0	±	0	+	+	+	0	+	+
Maltose.....	0	0	0	0	0	0	0	0	0	0	+	0	±	0
Lactose.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sucrose.....	0	0	0	0	+	0	0	0	0	0	0	±	0	0
Raffinose.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Inulin.....	0	0	0	0	0	0	0	0	0	0	+	0	+	0
Mannitol.....	0	0	+	0	0	0	+	0	+	+	+	0	+	+
Glycerol.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ethyl alcohol.....	0	0	+	0	+	0	+	0	+	+	+	+	+	+
Salicin.....	0	0	0	0	0	0	0	0	0	0	+	0	+	0
Sodium lactate.....	0	0	+	0	+	0	+	+	+	+	+	+	+	+
Sodium citrate.....	0	0	+	0	0	0	0	+	+	+	+	±	+	+
Sodium acetate.....	0	+	+	±	+	±	+	+	+	+	+	+	+	+

The symbols have the following meaning: + indicates definite utilization; ± indicates slow utilization; 0 indicates no evidence of utilization.

organisms studied. Glucose was utilized by all except the M-1 and bovine strains of *Myco. tuberculosis*. It is possibly significant that acetic acid is utilized by so many of the organisms. In the majority of cases the utilization of acetate was more prompt and yielded a better growth than was the case with glucose. Lactate was likewise utilized by a majority of the organisms. Varying degrees of utilization of the other carbon compounds

are shown. The more saprophytic species utilized a larger number of the carbon compounds studied than did the more parasitic forms. There are undoubtedly some variations in the degree of utilization of individual strains within the same species as is demonstrated by the three strains of human type tubercle bacillus studied. A similar variation was noted in the various strains of *Myco. leprae* reported in a previous article (1930).

TABLE 5
Comparison of results obtained in broth and synthetic media cultures

		MYCO. AVIUM	MYCO. LEPRAE 282	MYCO. CHELONEI	MYCO. RANAE	MYCO. FRIED- GENSIS
Glucose	{ Broth.....	±	+	+	+	+
	{ Synthetic media.....	0	+	+	+	+
Fructose	{ Broth.....	+	+	+	+	+
	{ Synthetic media.....	0	+	0	+	+
Arabinose	{ Broth.....	+	±	+	+	+
	{ Synthetic media.....	0	0	0	±	0
Galactose	{ Broth.....	0	±	0	0	+
	{ Synthetic media.....	0	0	0	±	±
Sucrose	{ Broth.....	+	+	+	0	+
	{ Synthetic media.....	0	+	0	0	±

For explanation of symbols see table 4.

DISCUSSION

In the work reported above the carbon compounds, the utilization of which were being studied, constituted the only carbon source in the medium. It is of interest to compare the results thus obtained with the findings when other carbon sources were also present. The results herein reported show some variations from those obtained with the same carbohydrates when plain broth constituted the basic medium (1930). All such variations are included in the comparative results given in table 5. In only one instance was utilization demonstrable in the synthetic media where it was not shown in the carbohydrate broth, namely galactose by *Myco. ranae*. On the other hand utilization was

demonstrated in several instances in carbohydrate broth cultures where it could not be demonstrated in the synthetic media. The latter was true more particularly with the slow growing organisms. For example no utilization of glucose, fructose, arabinose, or sucrose by *Myco. avium* or of fructose, arabinose, or sucrose by *Myco. chelonae* was shown in the above determinations in synthetic media while utilization of all of these was demonstrable in the broth cultures previously reported. The possibility is thus suggested that the fermentative action of the organisms is increased by the presence of other utilizable carbon sources. The evidence is too incomplete, however, to conclude that such is the case. An alternative hypothesis is that some of the carbohydrates can be utilized by the organisms except that the rate of cleavage is too slow to permit active metabolism, which latter is really all the method used for the work here reported would show. Until studied further, however, these suggested explanations must be regarded as pure hypotheses.

The varied utilization of carbon compounds by the organisms studied is undoubtedly of some differential value. For example *Myco. leprae* and *Myco. friburgensis* can be separated from the remainder of the group by their ability to utilize sucrose. They can be differentiated from each other on a basis of mannitol utilization by *Myco. leprae* and lack of utilization by *Myco. friburgensis*. Again, *Myco. berolinensis* and *Myco. stercusis* are differentiable from the remaining organisms studied by their ability to utilize inulin, maltose, and salicin; but there appears no way of distinguishing the two from each other from the results presented.

The above differential possibilities hold well enough for the particular organisms studied. The question arises, however, as to the constancy of the utilization by various strains of the same species of organisms. It was observed in the preceding report (1930) that some strains of *Myco. leprae* utilize sucrose whereas other strains will not. It was found that all strains studied which were of known human origin utilized sucrose; the strains of rat origin possessed no such ability. Possibly the two organisms should be classified as two separate species.

It is significant that in so far as the same carbohydrates and the same species of organisms were used the results reported in this paper are for the most part comparable to the results reported by Kondo and associates. Since a greater number of organisms and several additional carbon compounds were included in this work than were reported by the previous workers an absolute comparison of results cannot be made. However the reported results are at variance in only two instances. The former workers report no utilization of lactate by *Myco. chelonae* whereas I was able to demonstrate utilization following three months incubation of the cultures. There are also some discrepancies in compounds utilized by the human strains of *Myco. tuberculosis*. I was unable to demonstrate utilization of glucose by the M-1 strain or of acetate by the H-37 strain of the latter organism. Kondo et al. reported very slow utilization of glucose and definite utilization of acetate by the human strain they employed.

From the results obtained thus far, some doubt is therefore thrown upon the constancy of the utilization of the same carbon compound by the various strains of the same species of organisms. Especially is this shown to be the case with the human strain of *Myco. tuberculosis*. The slow growth of the pathogenic strains of this organism, even under optimal conditions for growth, introduces difficulties in determination of utilization by the methods here employed that undoubtedly plays some part and perhaps even a predominating part in the production of the variations encountered.

Attention should be called to the type of reaction changes observed in the synthetic media whenever utilization took place. In the case of the carbohydrates and alcohols the change was uniformly toward increased acidity. This contrasts strikingly with the uniform change towards increased alkalinity encountered when the organisms were grown in carbohydrate broth (1930). The discussion of the cause of these reaction changes will be considered in a subsequent paper. On the other hand wherever acetate, citrate, or lactate were utilized there was in each case a pronounced primary change in reaction to the alkaline with a subsequent decrease in alkalinity, the terminal reaction being near that at the time of inoculation. The primary change may be

attributed to the utilization and removal of the organic acid radicle of the salts of the organic acids studied. The causes of these reaction changes, however, have not been investigated.

SUMMARY

1. All organisms of the genus *Mycobacterium* included in this study possess the ability to obtain their carbon and energy supply from some carbohydrates, alcohols, and salts of organic acids, when these are present in a synthetic medium and constitute the sole carbon and energy source. The results of the utilization determinations are summarized in table 4.

2. Utilization of carbon compounds by various members of this group as determined by the methods here employed is of sufficiently varied nature to become of value as a means of differentiating some of the species comprising this genus.

3. In media containing salts of organic acids as the carbon source the reaction changes associated with growth of these organisms is a primary alkalinity followed by a secondary change back towards neutrality. When the media contain carbohydrates or alcohols the reaction change is toward progressive increase in acidity.

4. There were some cases in which utilization could not be demonstrated by the method here used where the same carbohydrates were shown to be utilized by the same organisms in the carbohydrate broth cultures.

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STUDIES ON CARBON METABOLISM OF ORGANISMS OF THE GENUS MYCOBACTERIUM

III. END PRODUCTS OF CARBOHYDRATE UTILIZATION AS DETERMINED IN SYNTHETIC MEDIA CULTURES

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Received for publication January 2, 1931

Fermentation of carbohydrates by the majority of bacteria is accompanied by the accumulation of acid cleavage products in the medium. The reaction of the medium in such cases thus changes towards increased acidity. It was pointed out in a previous report (1930) that such an accumulation of acid cleavage products in carbohydrate broth cultures of the *Mycobacteria* does not occur when carbohydrates are utilized. The cultures in all cases become progressively more alkaline regardless of the degree of carbohydrate utilization. A mechanism of utilization differing somewhat from the usual type, which is characterized by incomplete cleavage of the carbohydrate, was thus indicated. From the results obtained it appeared that whenever the *Mycobacteria* attack a carbohydrate molecule a complete cleavage results, the end products of such cleavage being carbon dioxide and water with possibly some of the carbon going into the construction of the bacterial cells.

When a study was made of reaction changes induced by growth of *Mycobacteria* in a synthetic medium it was found that when carbohydrates constituted the carbon source the reaction changed towards increased acidity (1931). The question thus arose whether or not acid cleavage products were formed from the carbohydrates under these methods of experimentation. The following experiments were performed in an effort to determine the fate of the carbon which was added in the form of a carbohydrate to a synthetic medium.

METHODS

A medium containing the following ingredients dissolved in distilled water was used for these determinations:

	<i>per cent</i>
NaCl.....	0.5
(NH ₄) ₂ SO ₄	0.5
MgSO ₄	0.005
K ₂ HPO ₄	0.4
Glucose.....	1.0

The various ingredients were weighed out into a volumetric flask which was then filled to the mark with distilled water. Immediately after solution of all the reagents had been effected the medium was sterilized by filtration and subsequently pipetted accurately, 10 cc. per tube. Incubation at 37°C. for one week followed in order to rule out the presence of contaminants.

It will be noted that potassium phosphate is added to twice the concentration used in the work previously reported (1931). This increases the buffer effect and thereby prevents too rapid an increase in acidity with its associated inhibition of growth.

The species of *Mycobacterium* studied are listed in the table of results. In addition several non-acid fast organisms were studied such as *B. coli*, *Staphylococcus aureus*, *B. typhosus*, *B. subtilis*, and *B. proteus*.

After inoculation the tubes were placed in 1 liter Florence flasks to which had just previously been added an accurately measured amount of 0.2 N barium hydroxide. The flask was stoppered tightly employing specially prepared rubber stoppers as used in the former work (1930). Uninoculated tubes of media in flasks containing the same amount of barium hydroxide were used as controls.

At the termination of incubation the following determinations were made:

1. The carbon dioxide liberated from the culture was determined by titration of the residual barium hydroxide employing thymolphthalein as indicator. The difference multiplied by the factor 4.4 gave the milligrams of carbon dioxide liberated. Since all the cultures were acid in reaction no carbon dioxide was

retained in the medium. Repeated determinations demonstrated that wherever the pH was below 7.0 there could be no carbon dioxide demonstrated in the culture medium.

2. The titratable acidity of the culture medium was next determined. One-tenth cubic centimeter of 0.1 per cent phenol red was added to each tube. In all cases the medium was definitely acid. Then, employing N/40 sodium hydroxide, the reaction of the culture was brought to that of the uninoculated control.

3. The medium in each tube was next diluted to 100 cc. with distilled water, centrifuged, and the supernatant fluid withdrawn to be used for the carbohydrate and ammonia determinations. These latter determinations on the supernatant were run exactly as outlined for the analogous work in which carbohydrate broth was employed, reported in a previous paper (1930).

4. The sediment contained in the centrifuge tube was resuspended in distilled water and recentrifuged five successive times to remove all minerals, then transferred to accurately weighed crucibles. Subsequently the crucibles were placed in a desiccator over concentrated sulphuric acid and dried to constant weight. The weight of the growth was thus determined.

The calculations were made as for the analagous work previously reported.

RESULTS

The results of one series of determinations are reported in table 1. Three cultures out of the series noted in the table were contaminated by a mold on the termination of the incubation. None of the controls, eight of which were used in these determinations, were contaminated. The determinations here reported typify the results obtained regardless of the duration of incubation or the percentage of the carbohydrate contained within the medium.

It will be noted that in the incubation period reported most of the glucose was utilized by all the Mycobacteria tested. The average glucose content of the control tubes as determined by the Shaffer-Hartman method was 0.926 per cent or 92.6 mgm. of glucose per 10 cc. of media. One hundred and thirty-six milligrams

TABLE 1
Results of quantitative determinations on synthetic media cultures

ORGANISMS	INCUBATION days	CATION DIOXIDE RECOVERED	GLUCOSE UTILIZED	CARBON DIOXIDE POSSIBLE	POSSIBLE CARBON DIOXIDE RE- COVERED	AVERAGE OF POS- SIBLE CO ₂ RE- COVERED	LITRATABLE ACID IN CC. N/40 NaOH	NH ₃ DECREASE IN TITRATION OF N/40 NaOH	WEIGHT OF ORGANISMS	CARBON IN ORGAN- ISMS (CALCULATED)	CARBON RECOVERED AS CO ₂	CARBON IN UN- UTILIZED CAR- BOHYDRATE	TOTAL CARBON ACCOUNTED FOR	TOTAL CARBON PRESENT IN MEDIUM
<i>Myc. tuberculosis (hominis)</i>	42	115	92.6	136	84.5		3.2	4.0						
	42	126	92.6	136	92.6		4.1	4.4						
	42	109	89.6	132	82.8	86.6	4.0	4.4	32.2	16.1	95.2	1.2	112.5	111.3
<i>Myc. berolinensis</i>	42	92	85.1	125	74.4		3.6	3.6						
	42	89	79.1	116	76.5		4.0	4.4	32	16	85.0	10.1	111.1	111.3
	42	131	85.9	126	103.0	84.9	3.7	4.0						
<i>Myc. friburgense</i>	42	99	80.1	118	84.0		3.7	4.4						
	42	113	92.6	136	83.1		3.7	3.6						
	42	124	62.6	136	91.1	86.1	3.9	4.0	33.3	16.7	91.6	5.0	113.3	111.3
<i>Myc. stercuris</i>	42	117	92.6	136	86.0		3.4	4.4						
	42	117	92.6	136	86.0	86.0	4.0	3.2	24.8	12.4	63.8	0.0	76.2	74.2
	42	Contaminated	Contaminated											
<i>Myc. phlei</i>	42	115	90.6	133	85.5	85.5	3.9	4.0	9.1	5.6	31.2	0.7	37.5	37.1
	42	Contaminated	Contaminated											
	42	Contaminated	Contaminated											
<i>B. proteus</i>	24	6.6	14.5	21	31.0	31.0	5.0	0.0						
Controls (average)	42	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	37.1		37.1

(92.6 multiplied by the factor 1.47) of carbon dioxide could have been produced from this amount of glucose were the carbon quantitatively converted to carbon dioxide. While the percentage of possible carbon dioxide recovered varies among the individual cultures of any one organism the averages as given in the column "average per cent of possible carbon dioxide recovered" are comparable.

The titratable acidity in cc. of $N/40$ sodium hydroxide in every case can be accounted for wholly on a basis of the ammonia decrease. It is to be remembered that the ammonia determination was run on but one fourth of the culture. An error of 0.1 cc. in the titrations would thus introduce an error of 0.4 cc. in the column "ammonia decrease in terms of cc. $N/40$ NaOH." Since ammonia constituted the only source of nitrogen in the media a decrease would be expected. As the ammonia is withdrawn from the compound ammonium sulphate free sulphuric acid would remain, thus accounting for the change in the reaction of the media towards increased acidity. These results demonstrate indirectly but rather conclusively that no cleavage products of the carbohydrate, which in any way tend to lower the pH, accumulate in the medium.

The composite weight of the organisms from all the cultures of each species of organism was determined. From the work of previous investigators (Kruse (1910)) it would appear that approximately 50 per cent of the weight of washed, dried acid-fast organisms is carbon. Employing this percentage the carbon contained in the organisms was approximated. In the next column in the table the mgm. of carbon recovered as carbon dioxide is given. This represents the total carbon dioxide recovered multiplied by $\frac{3}{11}$. In the next column the carbon remaining in the media as unutilized glucose is given. The total carbon accounted for is listed next, followed by the total carbon present in the uninoculated controls. It will be observed that the latter two figures in each case compare within at least 2 mgm. It would seem that this quite conclusively demonstrates that the carbon in the glucose is converted quantitatively into carbon dioxide or utilized in the synthesis of the bacterial cell. These

results definitely contraindicate the existence of any cleavage products of the glucose in the media.

The results obtained from determinations on one culture of *B. proteus* are given at the end of the table. These results are typical of those obtained with organisms of the colon-typhoid group and of staphylococci. It will be noted that only 6.6 mgm. of carbon dioxide were recovered while 14.5 mgm. of glucose were utilized from which 21.3 mgm. of carbon dioxide could have been produced. Thus 31 per cent of the possible carbon dioxide was recovered as compared to the 85 per cent in the case of the *Mycobacteria*. Also of significance is the recorded titratable acidity equivalent to 5.0 cc. of N/40 NaOH without any associated demonstrable ammonia decrease, which definitely indicates acid production from the carbohydrate. The growth of these organisms was so limited that a determination of the weight of the growth was not feasible.

DISCUSSION

The above work adds definite evidence to the conception that when organisms of the genus *Mycobacterium* utilize carbohydrates there is no associated accumulation of cleavage products in the media. The question of the process by which the utilization is accomplished is not clarified by the present work. It is significant that acetic and lactic acids are utilized possibly more readily than glucose (see preceding article). It is thus possible that these acids may be intermediate products in glucose metabolism and that they actually are present outside the cell but that at any given time they are present in such minute amounts as not to be demonstrable by the methods employed. Experiments employing "resting" organisms similar to those devised by J. H. Quastel (1928) and by Kendall et al. (1930) offer an excellent opportunity for further study of the mechanism of utilization of carbohydrates by these organisms.

SUMMARY

1. In glucose synthetic media cultures of the organisms of the genus *Mycobacterium* studied, all the carbon available in the

media was accounted for as carbon dioxide plus the carbon contained in the organisms, plus that remaining as unutilized carbohydrate.

2. The reaction changed toward increased acidity, accompanying growth of the Mycobacteria in the synthetic medium used. This reaction change can be accounted for completely on a basis of the removal of ammonia from the media. There is no evidence that any cleavage products of the carbohydrates accumulate in the media.

3. It would appear from all evidence at hand that the Mycobacteria utilize carbohydrates completely, no cleavage products accumulating in the media, the carbohydrate being oxidized completely to carbon dioxide and water.

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A COMPARISON OF COLORIMETRIC AND POTENTIOMETRIC METHODS FOR HYDROGEN ION DETERMINATION OF SOLID BACTERIAL MEDIA, USING A DILUTION METHOD BASED ON THE BUFFER EQUATION^{1,2}

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Received for publication December 5, 1930

INTRODUCTION

In our unpublished studies concerning the effect of the medium on the electrophoretic velocity of the organism grown thereon, it became necessary to measure the pH of solid bacterial media. In the light of the errors exhibited by indicators, the question arose as to whether the colorimetric method (which is most commonly used in the bacteriological laboratory because it has the advantage of simplicity) is sufficiently accurate for this purpose. Whenever highly accurate values are necessary, the colorimetric values should be verified by potentiometric determinations. Previously, this has been done with liquid but not with solid media.

Since we were dealing with a solid medium the question arose as to how to make pH determinations on such a medium potentiometrically. It was thought that if the medium was sufficiently buffered, it would be possible to do this by diluting the melted agar until it remained a liquid at room temperature, an idea suggested by buffer principles.

¹ This is a resumé of a thesis submitted by O. G. Jensen in partial fulfilment of the requirements for the degree of Master of Science in Agricultural and Biological Chemistry in the Graduate School of The Pennsylvania State College, 1929.

² Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station as Technical Paper No. 513.

HISTORICAL

For the fact that the colorimetric method is not always accurate, the reader is referred to Sörenson and Palitzsch (1913), and Clark and Lubs (1917). Jaumain (1925) reports an error of 0.8 pH when using bromthymol blue to determine the pH of a 1/50 concentration of blood serum. On the basis of this he recommends that the indicator method be avoided for determinations of protein-containing materials. Schlegel and Stueber (1927) call attention to the acid error in the use of bromthymol blue. The salt error has been studied thoroughly by Kolthoff (1918, 1925). He found bromthymol blue among the indicators exhibiting the smallest error. Sharp and McInerney (1926) state that it is not advisable to use indicators at the limit of their ranges unless the determination is checked by the use of an indicator whose limits are well within the observed pH.

Cullen (1922) introduced a colorimetric method for determining the pH of blood serum and plasma. He determined a factor, by the use of which, one can convert the colorimetric pH value obtained at room temperature on a sample which has been diluted to 20 times its original volume, to the actual value at 38°C., as determined by the hydrogen electrode. Hastings and Sendroy (1924) report that the correction used by Cullen is not necessary when the readings are made at body temperature. They find that the average difference under these conditions is only 0.003 pH, the colorimetric value being the higher. Johnston (1928) and Myers and Muntwyler (1928) discuss this point at greater length.

The hydrogen electrode can be used where turbidity and color interfere with indicators. The bacteriologist does not use potentiometric methods to a large extent; they are too cumbersome, and it is seldom that reactions need to be determined more accurately than the colorimetric method permits. In the light of the above statements, however, he needs to be aware of the fact that colorimetric and potentiometric values do not always agree, and therefore, he must ascertain whether or not such a difference does or does not exist, and whether or not he is justified in using the simpler colorimetric method.

Up to the present time there is but one record of an attempt to measure directly the hydrogen ion concentration of a solid bacterial medium by means of a hydrogen electrode. Radsimowska (1924) claims that he was able to get an excellent agreement between the results obtained with his electrode and with the ordinary U-form electrode of Michaelis in the usual buffer solutions of Sörenson, Michaelis, and others. He states that a special report will contain the results of using this electrode to determine the pH of a single bacterial colony.

Biilmann and Lund (1923), and Biilmann (1927) have shown that the hydrogen electrode can be replaced by the quinhydrone electrode.

Biilmann and Krarup (1924) have determined potential differences at various temperatures with the quinhydrone electrode, and have given us an equation for calculating the potential of the electrode in respect to a hydrogen electrode at "t" degrees temperature. Recently, Biilmann and his co-workers (1928) tested the accuracy of the quinhydrone electrode in phosphate solutions, and found that the quinhydrone electrode gave correct pH values up to 7.73. Bayer (1926) found that with most soils constant readings were easily and quickly obtained, but that in case of alkaline soils the readings must be taken immediately after adding the quinhydrone. Lester (1924), working with dairy products found good agreement in values obtained by the hydrogen and quinhydrone electrodes. Watson (1927) has measured the pH of Swiss cheese by means of the quinhydrone electrode, but found a rapid drift especially in "green" cheese.

The change in potential of the quinhydrone electrode has been studied by several investigators and it has been generally conceded that the drift is due to a slow change in the ratio between the concentration of the quinone and the hydroquinone. Several factors may operate to bring this about. If the material contains oxidizing or reducing substances, the hydroquinone may be oxidized to quinone or the quinone reduced to hydroquinone. Bayer (1928) thinks that one of the substances may be selectively adsorbed when soil suspensions are used. Hissink and van der Spek (1927) are of the opinion that the drift is due to the fact that equilibrium has not been established.

Considerable variation in opinion exists as to the best time to make the readings. Hetterschij and Hudig (1927) came to the conclusion that readings taken immediately after adding the quinhydrone are the best. On the other hand Hissink states that thirty minutes should be allowed to elapse before readings are made. Corran and Lewis (1924) and Cullen and Earle (1928) find that good agreement is obtained between the quinhydrone and hydrogen electrodes when readings are extrapolated to zero time.

The following are the buffer equations that apply to solutions of weak monobasic acids in the presence of their salts and to weak monoacidic bases in the presence of their salts respectively:

$$[\text{H}^+] = K_a \frac{[\text{acid}]}{[\text{salt}]}, [\text{OH}^-] = K_b \frac{[\text{base}]}{[\text{salt}]}$$

The equations state that, as long as the assumptions upon which their development is based are valid (see Michaelis, 1926, p. 44), the pH of such solutions will not change on dilution for it depends solely on the ratios $\frac{[\text{acid}]}{[\text{salt}]}$ and $\frac{[\text{base}]}{[\text{salt}]}$, provided temperature is kept constant.

Several investigators have taken advantage of the fact that buffered solutions can be diluted without great alteration of the hydrogen ion concentration. Brown (1924) assumed that the pH of culture fluids does not change upon dilution. To determine the hydrogen ion concentration of small amounts of material, he added one drop to a small quantity of water and made a colorimetric comparison. Hastings and Sendroy (1924) and Hastings, Sendroy, and Robson (1925) showed that blood and urine can be diluted to great advantage. Cullen (1922) diluted blood plasma to 20 times its original volume with physiological salt solution. The turbidity of milk is a great hindrance to colorimetric determinations and for this reason Sharp and McInerney (1926) have studied the possibility of diluting the milk and thus removing the difficulty. In order to determine the dilution factor they plotted the pH of the original milk and of the milk diluted to various volumes against the logarithm of the volume

to which the milk was diluted. The curves obtained were invariably straight up to a volume dilution of 20 times. At this dilution the correction factor is -0.54 in order to bring the pH to the original value. This factor applies only to milk whose original pH is between 6.0 and 7.4. When the milk is as acid as pH 5.0 the factor diminishes to 0.22. Kolthoff (1928) states that the effect of dilution on the pH of buffer mixtures can be calculated.

THE PROBLEM

In order to determine whether or not colorimetric methods are suitable for the determination of the pH of solid bacterial media, it becomes necessary to determine pH potentiometrically. Instances have been cited to show that colorimetric methods may be in error and that it has been found advantageous to dilute blood, milk, and urine, and to apply a correction factor for the accompanying change in pH. In view of the nature of the constituents of nutrient agar, it seemed probable that its buffer capacity would be large enough to permit dilution to the point where it would remain liquid and the use of potentiometric methods would become feasible.

The hydrogen electrode is easily poisoned by protein-containing material, thus requiring frequent replatinizations. On the other hand, the quinhydrone electrode requires no platinizing and is simple to manipulate. For this reason, it was decided to determine the applicability of the quinhydrone electrode to these studies.

The problem consists of two parts: (1) A justification for the dilution; (2) a comparison of colorimetric and potentiometric methods, including both the hydrogen and quinhydrone electrode.

EXPERIMENTAL METHODS AND APPARATUS

The determinations were carried out on a medium made by dissolving 31 grams of dehydrated Bacto Nutrient Agar (Bacto-beef extract, 3 grams; Bacto-peptone, 5 grams; Bacto-agar, 15 grams, and sodium chloride 8 grams) and 1000 cc. of distilled water and autoclaving for twenty minutes at 15 pounds of pressure. Before the medium solidified, portions were diluted to 3, 5, 7, 10, and 15 times their original volumes, the distilled water

used having a pH of 6.0 ± 0.2 . Colorimetric determinations were made upon the undiluted medium and upon each of the diluted portions. Potentiometric determinations were made upon the portions that remained liquid. These were the portions diluted 5, 7, 10, and 15 times. In order to preclude any possibility of an error due to changes in pH with time, the determinations were made in alternating order.

Bromthymol blue, purchased from the LaMotte Chemical Products Company, was used for all of the colorimetric determinations. The set of comparison tubes was purchased especially for this study and kept in a refrigerator when not in use. The comparisons were made by the light from a Palo Daylight Lamp, thus avoiding the danger of dichromatism.

The apparatus for the potentiometric determinations centered about a Leeds and Northrup Type K Potentiometer, the null point instrument being an enclosed lamp galvanometer with a sensitivity of 0.025 microampere per millimeter scale reading. The known E.M.F. was furnished by an Eppley Cadmium Cell of the unsaturated Weston type. Its potential was 1.01878 volts at 20°C. No correction was made for temperature changes as the temperature coefficient is negligible between 15° and 35°C.

The hydrogen electrodes were of the Bailey type (1920). Two of the quinhydrone electrodes (nos. 2 and 3) consisted of platinum foils, approximately 4 x 8 mm. in size, while the other (no. 1) was made of 4 cm. of (24 gauge) platinum wire. A saturated calomel cell was the reference electrode. The hydrogen which was supplied from a cylinder was purified by passing it successively through solutions of alkaline pyrogallol, alkaline potassium permanganate, and saturated mercuric chloride. The quinhydrone was purchased from the Research Laboratory of the Eastman Kodak Company.

Each day, before any determinations were made, the standard cell was checked against a new cell of the same type which was kept as a standard. Following this the electrodes were filled with a standardized buffer solution and measurements taken to insure that the reference cell and each of the electrodes was in good condition. Any hydrogen electrode that was unduly slug-

gish or that gave readings more than 0.02 pH in error was discarded. The quinhydrone electrodes were cleaned in chromic acid cleaning solution and flamed with an alcohol lamp.

PRESENTATION OF DATA

It has been stated that many investigators have observed a drift in the potential of the quinhydrone electrode. We have found a similar change with nutrient agar. In order to determine the exact nature of the drift, we have thought it advisable to observe it over an extended period of time. Table 1 gives the

TABLE 1
Showing drift in the potential of the quinhydrone electrode

TIME*	E.M.F.	TIME*	E.M.F.
1	0.0261	20	0.0615
2	0.0297	23	0.0641
3	0.0328	25	0.0656
4	0.0359	30	0.0692
5	0.0384	35	0.0721
8	0.0449	40	0.0751
10	0.0488	45	0.0775
11	0.0504	50	0.0800
14	0.0547	55	0.0821
15	0.0560	60	0.0842
17	0.0583		

* Time in minutes.

results on a sample diluted to 10 times its original volume. Time is counted from the time of addition of the quinhydrone.

The data of table 1 are shown graphically in figure 1. It is evident that the curve over the interval from zero to five minutes does not deviate greatly from a straight line. For this reason the extrapolated values, tables 2 and 3, are made on the assumption that for the first five minutes the drift is practically a linear function of the time.

The pH values using the Bailey electrode were calculated as usual whereas those for the quinhydrone determinations were made by the method suggested by Büllmann and Krarup (1924),

except in one set of data which were calculated according to Cullen *et al.* (1925, 1928).

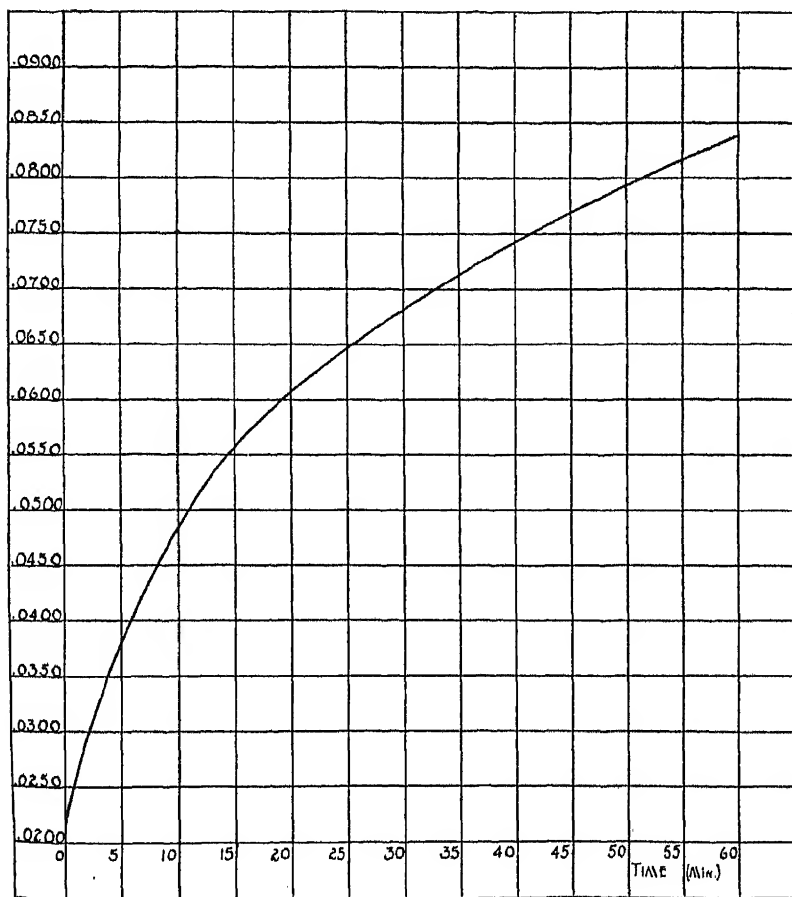


FIG. 1. THE DRIFT IN POTENTIAL OF THE QUINHYDRONE ELECTRODE

TABLE 2

A typical set of data obtained at the various dilutions by the three methods under consideration, at 25°C.

DILUTION	COLORIMETRIC pH	QUINHYDRONE ELECTRODE							HYDROGEN ELECTRODE—BAILEY		
		Electrode number	E.M.F. at 1 minute intervals after adding quinhydrone						Electrode number	E.M.F.	pH
			0	1	2	3	4	5			
None	7.2										
1:3	7.2										
1:5	7.2	1	0.0302	0.0324	0.0351	0.0372	0.0391	0.0411	7.15	1	0.6728 7.21
1:5		2	0.0293	0.0316	0.0333	0.0365	0.0396	0.0407	7.16	2	0.6731 7.22
1:5		3	0.0299	0.0325	0.0355	0.0384	0.0405	0.0430	7.15	3	0.6736 7.23
									7.15*		7.22*
1:7	7.2	1	0.0355	0.0375	0.0387	0.0407	0.0422	0.0440	7.06	1	0.6717 7.20
1:7		2	0.0346	0.0366	0.0387	0.0403	0.0426	0.0444	7.07	2	0.6730 7.22
1:7		3	0.0355	0.0375	0.0397	0.0418	0.0427	0.0454	7.06	3	0.6731 7.22
									7.06		7.21
1:10	7.1	1	0.0377	0.0393	0.0410	0.0424	0.0442	0.0458	7.02	1	0.6714 7.19
1:10		2	0.0378	0.0394	0.0411	0.0427	0.0443	0.0456	7.02	2	0.6716 7.19
1:10		3	0.0385	0.0402	0.0417	0.0434	0.0445	0.0470	7.01	3	0.6720 7.20
									7.02		7.19
1:15	6.9	1	0.0503	0.0515	0.0533	0.0544	0.0550	0.0561	6.81	1	0.6614 7.02
1:15		2	0.0504	0.0516	0.0528	0.0541	0.0553	0.0562	6.81	2	0.6628 7.05
1:15		3	0.0515	0.0525		0.0546	0.0556	0.0565	6.79	3	0.6616 7.03
									6.80		7.03

* Average.

TABLE 3

Presenting average values from five sets of determinations made as per table 2

The average values are average hydrogen ion concentrations expressed in terms of pH.

METHOD	COLORIMETRIC						QUINHYDRONE ELECTRODE				HYDROGEN ELECTRODE—BAILEY			
	None	1:3	1:5	1:7	1:10	1:15	1:5	1:7	1:10	1:15	1:5	1:7	1:10	1:15
Dilution.....														
Set 1.....	7.2	7.2	7.2	7.2	7.1	6.9	7.15	7.06	7.02	6.80	7.22	7.21	7.19	7.03
Set 2.....	7.2	7.2	7.2	7.2	7.1	6.8	7.10	7.16	7.04	6.73	7.17	7.23	7.17	6.95
Set 3.....	7.2	7.2	7.2	7.2	7.0	6.9	7.01	7.03	6.93	6.84	7.16	7.17	7.15	7.04
Set 4.....	7.2	7.2	7.2	7.2	7.2	7.2	7.13	7.13	7.16	7.13	7.33	7.36	7.36	7.33
Set 5.....	7.2	7.2	7.2	7.2	7.2	7.2	7.23	7.20	7.18	7.14	7.28	7.31	7.31	7.31
Average.....	7.2	7.2	7.2	7.2	7.1	7.0	7.12	7.11	7.06	6.90	7.23	7.25	7.23	7.11

DISCUSSION OF RESULTS

It is quite evident that the original medium can be diluted to at least seven times its initial volume without a change in pH. From a knowledge of the buffer equation one would expect that dilution could not be continued indefinitely without a change in pH. Dilution of the agar to 10 and 15 times its initial volume caused a change in pH, as shown by all the methods used.

The drift in potential of the quinhydrone electrode was remarkably regular, successive readings giving values farther removed from the true hydrogen ion concentration. It is thought that the drift was due to a slow oxidation of the hydroquinone. However, even after excluding the effect of the drift by extrapolation to zero time, our values show that the quinhydrone electrode gives values which average 0.16 pH lower than those found with the hydrogen (Bailey) electrode. This intimates that there is another source of error not directly associated with the oxidation of the hydroquinone.

It was originally planned to use the bubbling electrode along with the Bailey type (1920) as a further means of comparison, but as consistent results could not be obtained, no data have been reported for it.

Clark and Lubs (1917) found that, for a liquid medium of about the same protein and salt content as the medium with which we worked, the colorimetric values were on the average 0.1 pH lower than the hydrogen electrode values. Our hydrogen electrode and colorimetric values agree well, showing that bromthymol blue does not exhibit either salt or protein errors in nutrient agar, and that the colorimetric method as ordinarily used gives accurate results.

SUMMARY AND CONCLUSIONS

A method, based on the buffer equation, has been devised for the determination of the pH of solid bacterial media, as it has been shown that the buffering power of nutrient agar is sufficient to permit a seven-fold dilution without a change in pH. At this dilution, the medium remains liquid, and the use of potentiometric methods is feasible.

Due to the cumbersomeness of the hydrogen electrode, the applicability of the quinhydrone electrode to pH determinations of nutrient agar has been determined. An effort has been made to eliminate the effect of the drift of the quinhydrone electrode by extrapolation of the values observed at successive intervals of time.

The average results of 60 parallel determinations indicate that the quinhydrone electrode gives values 0.16 pH lower than the hydrogen electrode.

It has been shown that bromthymol blue is an accurate indicator in nutrient agar, and that the colorimetric method agrees with the potentiometric method using the Bailey electrode.

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DO BROTH CULTURE FILTRATES CONTAIN A BACTERIAL GROWTH-INHIBITING SUBSTANCE?

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Presented before the Society of American Bacteriologists December 30, 1930

In 1923, Besredka reported that staphylococcus broth culture filtrates contain a substance which inhibits the growth of the homologous organism, but which exerts little or no effect upon the development of heterologous bacteria. He claimed, further, that this substance, to which he gave the name "antivirus," originates as a result of the disintegration of the bacterial cell and is diffused into the surrounding medium. These conceptions are summarized in the following quotations:

Quand on filtre sur bougie une culture de Staphylocoques en bouillon, âgé de 18 jours, on obtient un liquide qui, au premier abord, ne diffère pas sensiblement du bouillon ordinaire: ensemencé avec des microbes variés, ce liquide donne des cultures pouvant presque rivaliser, quant à leur richesse, avec du bouillon normal. . . . Seuls, les Staphylocoques—quelle qu'en soit l'origine—réensemencés dans ce liquide, n'y poussent pas: ils conservent leur vitalité, mais sont incapable d'y faire souche. . . . La culture filtrée renferme donc une substance qui paralyse l'activité des Staphylocoques, aussi bien in vivo que in vitro. . . . Enfin, comme l'indique son mode d'obtention, cette substance a le pouvoir de diffuser dans le milieu ambiant.

Besredka's belief that other bacteria can be used in the preparation of similar filtrates is indicated in a later publication (1930) as follows: "Les mêmes principes président à la préparation des autres antivirus—streptococcique, colibacillaire, typhique ou tuberculeux; seuls varient dans ces cas les milieux de culture et quelquefois la durée du séjour à l'étuve." The following excerpt states clearly that "antivirus" has its origin in the bacterial cell

(1925): "La dislocation des corps microbiens a pour résultat la mise en liberté d'antivirus;" Other methods of preparation are also cited (1930), whereby the liberation may be accomplished by autolysis, rapid disintegration, etc.

Weichardt (1927) found that undiluted filtrates were inhibitory and believed that the action was due to the presence of protein split products in certain concentrations and therefore non-specific. He showed, further, that the addition of the filtrates, in dilutions of 1:10 and 1:100, to a synthetic medium resulted in growth stimulation. Ninni and Molinari (1928) reported also that filtrates were inhibitory and reached the conclusion that the effect was due to products of protein decomposition. After adding 2 parts of ordinary broth to the filtrate they observed that there was normal development of the organisms. When broth and filtrate were diluted equally with water and when the filtrate was added to equal amounts of ordinary broth, the growth was less than that in normal broth. As a result, they believed that the inhibitory effect was not due, except in a small degree, to a diminution of nutritive materials. Their investigations, therefore, led them to conclude that the inhibitory effect of the filtrates was in direct relation to the quantity of products formed by protein decomposition, and in inverse relation to the nutritive needs of the organisms inoculated. In some previous work, the writer (1929) inoculated *Staphylococcus aureus* and *Escherichia communior* into homologous and heterologous filtrates and noted that the growth of the organisms under both conditions was much less than in normal broth. Broth digested by the use of non-bacterial enzymes also failed to support growth of *Esch. communior* and *Staph. aureus* to the same degree as the original bouillon. The interpretations of these results appeared to lend support to the conclusions of Ninni and Molinari. Schweinburg (1928), however, after adding normal broth to culture filtrates and observing abundant growth, reached the conclusion that the failure of organisms to grow in homologous filtrates is due to an exhaustion of nutritive materials. Chaillot (1930), on the other hand, reported inhibition of staphylococcus growth in a mixture of 5 cc. of broth and 5 cc. of the homologous filtrate. He stated

that, " . . . l'action empêchant d'un antivirus n'est nullement la conséquence de l'appauvrissement du milieu en principes nutritives."

In the literature cited, there appear to be three conceptions as to the nature of the effect of broth culture filtrates on bacterial growth: (1) that an active inhibitory substance ("antivirus") is liberated from the bacterial cells (Besredka); (2) that inhibitory products are formed as a result of protein decomposition (Weichardt, Ninni and Molinari); and (3) that the effect is due to an exhaustion or diminution of food materials (Schweinburg).

Due to these conflicting reports, it was deemed advisable to obtain further experimental evidence upon the problem. The possibility that the entire phenomenon may be due to simple dilution or exhaustion of nutrient material led to the experiments described in the present paper. These investigations aimed, not only to control the question of dilution of nutrient material, but attempted also to separate the elements in the cells from those in the medium, and to determine the possible presence of inhibitory substances in each.

METHODS

In general, the technic used in testing the different preparations for inhibition of bacterial growth was as follows: Varying amounts of the filtrate to be tested were added to a series of seven test tubes containing nutrient broth; the final concentrations of filtrate in a series ranged from 5 to 95 per cent; a tube containing broth only, and one containing filtrate only were included in each series. Strict asepsis was always observed. The organism being tested for growth was then inoculated, and growth recorded the following day. Growth of the same organism in broth containing the above proportions of distilled water or saline was noted at the same time.

Estimations of the growth were made by comparing the turbidity of the various tubes with that in tubes of plain broth. In the earlier part of the work such readings were checked by use of the Gates apparatus (1920), and by direct counts. Results were compared with those obtained by a disinterested member of

the staff and were satisfactory. The readings given, therefore, are those made by turbidity comparisons, and are recorded as follows: 3+ = normal growth; 1+ sl. = slight growth; \pm = growth doubtful; - = no visible growth.

The *broth* employed in all experiments was the usual laboratory meat extract bouillon (3 grams Liebig's beef extract, 10 grams Bacto-peptone, 5 grams sodium chloride, c.p., and 1000 cc. distilled water), adjusted to pH 7.2 to 7.4, and autoclaved at 15 pounds pressure for fifteen minutes. When necessary, the broth was passed through filter paper before sterilization.

The *reactions* of all broths and filtrates were adjusted to pH 7.2 to 7.4 before use.

Berkefeld N *filters* were used in all cases.

Controls for sterility of broth and filtrate, and for normal growth in plain broth were included in each series.

The *cultures* of *Escherichia coli* and *Staphylococcus albus* were obtained from the department stock collection. For inoculation, three to seven-hour broth cultures were transferred by pipettes. One drop of the young culture was added to each tube, care being taken to avoid contamination. No visible turbidity resulted from the inoculation of this amount of culture.

PREPARATION OF FILTRATES

1. *Filtrates of altered broth*

Artificially digested broth was obtained by adding 0.06 per cent dry pancreatin (Merck's U.S.P.), 0.14 per cent Na_2CO_3 , and 0.09 per cent toluene to bouillon. The mixture was incubated at 37.5°C. for periods varying from seven to fourteen days. After incubation, the material was heated for thirty minutes at 100°C. to expel the toluene and to destroy enzymes, made up to the original volume with distilled water, and then passed through a Berkefeld filter. Undigested mixture was heated, filtered, and used as a control for normal growth.

Culture filtrates were prepared by inoculating flasks of broth with *Esch. coli*, and incubating, heating, and filtering simultaneously with the pancreatinized broth.

2. Filtrates of bacterial cell solutions

Solutions of bacterial cells were prepared in three ways: (a) by autolysis; (b) by mechanical shaking; and (c) by dissolution in sodium hydroxide. In all cases the bacterial cells were obtained by growing large quantities on plain agar for twenty-four hours at 37.5°C. The growth was washed off with 0.85 per cent salt solution, centrifuged and washed three times with saline, then resuspended in the solution desired. After solution of the cells had been brought about, the preparations were passed through Berkefeld filters. Biuret tests showed the presence of protein in the filtrates.

a. Autolysates were obtained by placing 1 per cent suspensions of *Esch. coli* and *Staph. albus* in sterile distilled water and saline. The preparations were allowed to incubate for seventy-three days at 37.5°C. They were shaken at frequent intervals to hasten disintegration. It has been shown that autolysis of bacteria may occur in two to ten days (Rettger (1904)). Streak plates were made from each suspension at frequent intervals. Plates made on the last day of incubation showed marked decreases in the numbers of viable organisms. Suspensions in saline showed greater decreases than those in distilled water.

b. One per cent suspensions of *Esch. coli* and *Staph. albus* were placed in a shaking machine and shaken for eighteen hours with glass beads. Stained preparations of the centrifuged debris showed very few intact cells, indicating that most of the bacteria had undergone disintegration.

c. A 1 per cent suspension of *Esch. coli* was made up in a buffered saline solution. The suspension was then treated by adding 5 per cent by volume of 15 per cent NaOH and the mixture heated over the direct flame until maximum clearing occurred. Plain broth, in buffered solution, and buffered saline solution were treated simultaneously with the coli suspension. It was found necessary to use buffered solutions as previous work showed that the reactions of such preparations were not stable. The buffer used was a mixture of Na_2HPO_4 and NaH_2PO_4 in a final concentration of 0.2 M. This solution was isotonic by calculation.

EXPERIMENTAL DATA

In a preliminary experiment, it was shown that pancreatin-digested broth and broth cultures of *Esch. coli* are so altered during two to four days' incubation that their filtrates fail to support the growth of either *Esch. coli* or *Staph. albus* to the same degree as normal bouillon. In the case of the coli culture filtrate this phenomenon appears between the first and second days with regard to the homologous organism, and between the second and fourth days for the heterologous organism. There is an almost complete absence of visible growth of both organisms in a filtrate obtained after seven days' incubation of the coli broth culture. Growth of *Esch. coli* and *Staph. albus* in filtrates from two- to four-day pancreatin-digested broth is definitely less than in normal broth or undigested pancreatinized broth. The lack of ability to support growth exhibited in a filtrate taken after seven days' digestion by pancreatin is not as marked as in the case of the coli culture filtrate, but is much more evident than at the end of the fourth day. Growth of both organisms in undigested pancreatin broth was equal to or even more abundant than that in plain broth.

The results of this experiment suggest that the failure of organisms to grow in broth culture filtrates is not due to a specific factor; they lead to the assumption that the effect is not necessarily dependent upon bacterial growth, but to an alteration of proteins in the menstruum as suggested by Ninni and Molinari (1928).

If no actually bacteriostatic substance is present, the addition of the filtrates in varying amounts to nutrient broth should result in no greater decrease in growth than the addition of ordinary distilled water. In other words, if the phenomenon is due to an exhaustion of food materials, the filtrates when added to broth should act merely as a diluent of the nutrient material. The following experiments were devised to determine this point.

EFFECT OF FILTRATES OF ALTERED BROTH

Filtrates of a fourteen-day coli broth culture and of fourteen-day pancreatin-digested broth were prepared as previously

described. These were added to nutrient broth in proportions varying from 5 to 95 per cent. Similar series were prepared by the addition of sterile distilled water. The various series of tubes

TABLE 1

Effect of addition of broth filtrates and distilled water on growth of Esch. coli and Staph. albus in nutrient broth

ORGANISM INOCULATED	SUBSTANCE ADDED TO BROTH	PERCENTAGE OF SUBSTANCES ADDED TO BROTH								
		Broth only	5 per cent	25 per cent	35 per cent	50 per cent	65 per cent	75 per cent	95 per cent	Filtrate only
<i>Esch. coli</i> , homologous	Coli broth culture filtrate	3+	3+	2-3+	2+	1-2+	1+ sl.	1+ sl.	1+ very sl.	1+ very sl.
	Pancreatin digested broth filtrate	3+	3+	3+	3+	3+	2-3+	2+	1+	1+ very sl.
	Distilled water	3+	3+	2-3+	2-3+	1-2+	1-2+	1+ sl.	± to -	-
<i>Staph. albus</i>	Coli broth culture filtrate	3+	3+	3+	3+	3+	3+	1+ sl.	1+ sl.	1+ sl.
	Pancreatin digested broth filtrate	3+	3+	3+	3+	3+	3+	2-3+	2+	1+
	Distilled water	3+	3+	3+	2-3+	2+	2+	1-2+	1+ sl.	-
<i>Esch. coli</i> , heterologous	Coli broth culture filtrate	3+	3+	3+	3+	2-3+	2+	1-2+	1+ sl.	1+ very sl.
	Pancreatin digested broth filtrate	3+	3+	3+	2-3+	2+	2+	1+	1+	1+ very sl.
	Distilled water	3+	3+	2-3+	2-3+	2+	2+	1+	± to -	-

were inoculated with *Esch. coli* and *Staph. albus*. A third set of tubes was inoculated with a heterologous strain of *Esch. coli*. The results were observed the following day after incubation at 37.5°C., and are recorded in table 1.

It will be noted, in this table, that the decrease of growth caused by the addition of the broth culture filtrate was no greater than that effected by dilution of broth with ordinary distilled water. The same observation can be made with regard to the use of pancreatin-digested broth. It is also important to note that the homologous organism does not grow as well in broth diluted with the culture filtrate as do either of the heterologous organisms. This may be explained by assuming that organisms vary in their food requirements as regards amount and composition. Thus, it follows that a filtrate of a broth culture in which a given organism has been grown will contain less nourishment for the homologous species than for the heterologous type. The results shown in table 1 confirm this conception.

The results of this experiment fail to support the claim that broth culture filtrates contain a specific inhibitory agent originating as a result of disintegration of bacterial cells. They suggest, on the other hand, that the failure of organisms to grow in such preparations may be due to an exhaustion of necessary food substances.

EFFECT OF FILTRATES OF BACTERIAL CELL SOLUTIONS

It has been stated that the disintegration of bacterial cells results in the liberation, into the surrounding medium, of a substance capable of inhibiting the growth of the corresponding organisms. If this is true, filtrates of bacterial cell solutions added to nutrient broth should exhibit the phenomenon. Filtrates of the cell solutions previously described were accordingly added to broth in the manner employed in the preceding experiment. The results are shown in table 2.

The results given in this table show that no inhibitory agent was present in the cell solutions used. While there is a diminution in the growth of the organisms as the percentage of filtrate increases, the decrease is comparable to, or even less than, that exhibited when plain distilled water or saline is used in the same proportions. The experiment furnishes additional evidence against the assumption that the failure of organisms to grow in culture filtrates is due to products of cell disintegration.

TABLE 2

Effect of addition of filtrates of bacterial cell solutions on growth of homologous organisms in nutrient broth

ORGANISM INOCU- LATED	SUBSTANCE ADDED TO BROTH	PERCENTAGES OF SUBSTANCES ADDED TO BROTH								
		Broth only	5 per cent	25 per cent	35 per cent	50 per cent	65 per cent	75 per cent	95 per cent	Filtrate only
<i>Staph. albus</i>	Staph. autolysate in distilled water	3+	3+	3+	3+	2-3+	1-2+	1-2+	±	—
	Staph. autolysate in saline	3+	3+	3+	3+	2-3+	2+	2+	±	—
	Plain distilled water	3+	3+	3+	2-3+	2-3+	2+	1+	±	—
<i>Esch. coli</i>	Coli autolysate in distilled water	3+	3+	3+	3+	3+	2-3+	1+	± to +	—
	Coli autolysate in saline	3+	3+	3+	3+	2-3+	2+	1-2+	± to +	—
	Plain distilled water	3+	3+	2-3+	2+	2+	2+	1+	—	—
<i>Staph. albus</i>	Shaken staph. cell solution	3+	3+	3+	3+	2-3+	2-3+	1+	±	—
	Saline	3+	3+	3+	2-3+	2-3+	2+	1+	1+ very sl.	—
<i>Esch. coli</i>	Shaken coli cell solution	3+	3+	3+	2-3+	2+	1-2+	1+	1+ very sl.	± to —
	Saline	3+	3+	3+	2-3+	2+	1-2+	1+	1+ very sl.	—
<i>Esch. coli</i>	NaOH dissolved coli cell solution	3+	3+	2-3+	2-3+	2+	2+	1+	± to —	± to —
	NaOH treated broth	3+	3+	2-3+	2-3+	2-3+	2+	1-2+	1+ sl.	± to —
	NaOH treated saline	3+	3+	2-3+	2-3+	2+	1-2+	1+	± to —	± to —

The experiments described in this paper were repeated a sufficient number of times to rule out experimental error. Results were always comparable to those cited.

DISCUSSION

The fact that broth culture filtrates fail to support normal growth of bacteria is well known. Besredka (1923, 1930) has attributed this effect to the hypothetical presence of an inhibitory substance. He believes that this property is specific for the organism concerned, and arises as a result of disintegration of the bacterial cell. Ninni and Molinari (1928), on the other hand, have reported conflicting observations. Their evidence led them to conclude that the failure of organisms to grow in broth culture filtrates was due to an accumulation of products of proteolytic decomposition of the medium. They believe, further, that the effect is not specific, and varies according to the digestive activity of the organism used. These authors did not deny the existence of inhibitory properties in culture filtrates.

Evidence in support of these conceptions depends, in part, upon the following considerations: (1) It must be shown that broth culture filtrates will support the growth of certain heterologous organisms but not of homologous bacteria; (2) the presence of an actively bacteriostatic substance in such filtrates must be demonstrated; (3) preparations of disintegrated bacterial cells should inhibit the growth of the homologous organisms; (4) diminution of bacterial growth in nutrient broth diluted by the filtrates should be appreciably greater than that following the addition of water or saline in the same proportions.

The results of experiments described in this paper show that: (1) Broth, digested by pancreatin under the conditions described, did not support the normal growth of *Esch. coli* or *Staph. albus*; (2) broth culture filtrates of the organisms used failed to support normal growth of homologous and certain heterologous bacteria; (3) filtrates of solutions of disintegrated bacterial cells caused a diminution in growth of homologous organisms as the concentrations of filtrates increased; (4) in no case did the decrease in growth of organisms in broth diluted with culture filtrates or cell

solution filtrates exceed that exhibited when broth was diluted in the same proportions with distilled water or saline; in most instances the growth was less under the latter conditions. These findings disagree with Besredka's claims regarding the presence of an inhibitory agent in broth culture filtrates,—they lead to a denial of the existence of such a factor. There is a disagreement also with the report of Ninni and Molinari in so far as the presence of an inhibitory substance is concerned, but agreement with those authors in explaining the *apparent* inhibitory effect upon the basis of an alteration of proteins in the medium. The work of Butterfield (1929) on the relation between food concentration in liquid media and bacterial growth is of interest in connection with the observations reported in this paper. He found that an increase in food concentration, within certain limits, resulted in an increase in the numbers of organisms which developed.

These observations have a bearing on certain conceptions of local immunization. Besredka believes that broth culture filtrates possess local immunizing properties specific for the organism grown in the bouillon. Experimental results published by Gay and Morrison (1923), Freedlander and Toomey (1928), and others, indicate that the power of such filtrates to stimulate the production of local immunity is not specific. The effect is rather one of local response to irritants of a non-specific nature. The local immunizing effect of certain filtrates described in this paper remains to be determined.

CONCLUSIONS

The results of experiments described in this paper do not support the observations of Besredka that broth culture filtrates contain a bacterial growth-inhibiting substance liberated from the microbial cell.

When filtrates, prepared from broth cultures, pancreatin-digested broth, or bacterial cell solutions, are added in varying proportions to nutrient broth, the resulting diminution in growth of inoculated organisms is comparable to, or even less than, that observed when distilled water (or saline) is used as the diluent.

It appears to the writer, therefore, that the effect of culture

filtrates on the growth of bacteria is due to an alteration of necessary nutrient materials rather than to the presence of a truly inhibitory agent.

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COLI-AEROGENES DIFFERENTIATION IN WATER ANALYSIS

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Received for publication, December 31, 1930

The problem of differentiating fecal from non-fecal members of the coli-aerogenes group in water analysis is still in a chaotic state. Rogers, Clark and Davis (1914) first showed that the coli-aerogenes group could be divided into two sections depending upon the ratio of the carbon dioxide and hydrogen formed in glucose fermentation. Clark and Lubs (1915) showed that the gas ratios of the organisms correlated with the hydrogen ion concentration produced in a suitable culture medium and proposed the methyl red test. Levine (1916) demonstrated the high correlation between the methyl red test and the Voges-Proskauer reaction.

Following this, many workers, Rogers, Clark and Evans (1915), Chen and Rettger (1920), Wood (1920), Levine (1921), Koser (1924), Levine and Linton (1924), Jordan (1926), Tonney and Noble (1929) and others studied the "coliform" organisms isolated from soils and grains and from the feces of man and animals. The work of these investigators has well established the fact that the low-gas-ratio, methyl-red-positive, Voges-Proskauer-negative, *Bact. coli* section predominates in the feces of man and animals and that the high-gas-ratio, methyl-red-negative, Voges-Proskauer-positive, *Bact. aerogenes* section is relatively rare in feces and is common in soils and grains.

The source of the two sections of the coli-aerogenes group having been established, it would seem to be a simple problem to correlate the incidence of the *Bact. coli* and *Bact. aerogenes* sections to water quality. An examination of some of the work done to this end shows that the problem is not simple and that

the results have not been entirely successful. Winslow and Cohen (1918) found that the percentage of aerogenes strains isolated were as low from waters of unpolluted character as from waters of polluted character. Greenfield (1916) reported that 70 per cent of the cultures isolated from the surface waters of Kansas were of fecal origin. She also reported perfect correlation between the methyl-red and the Voges-Proskauer reactions. Perry and Monfort (1921) reported a number of cultures which were isolated from natural waters in Illinois to be irregular in respect to their methyl-red and Voges-Proskauer reactions. They considered such forms as intermediate or transitory in relation to the accepted coli or aerogenes types. They cited the work of Berrier, McCrady and Lafrenière (1916), Levine and Johnson (1916), Burton and Rettger (1916) and Bixley and Bartow (1917), who also found anomalous strains. Young and Greenfield (1923) concluded that the difference between soil and fecal strains was of little practical significance to the sanitarian and would not help him in judging the proximity of the source of contamination. Koser (1924) found the proportion of methyl-red-positive cultures to be almost the same for polluted and unpolluted water. Koser (1926) also reported some atypical or irregular cultures isolated from soils. Lewis (1926) isolated a large percentage of fecal type cultures from the waters of fissure springs remote from pollution. Clark (1927) is of the opinion that until further information is gained any member of the coli-aerogenes group should be reported as *Bact. coli*. Bahlman and Sohn (1924), Bardsley (1926) and Lewis and Pittman (1928) all made important differential studies on large numbers of organisms isolated from water. Their results are strikingly similar. They all obtained atypical or intermediate cultures and their studies indicate further that differentiation in routine work is not simple and that the results are not clear cut and are difficult or impossible to interpret.

The above studies were almost invariably made on cultures isolated on solid media after preliminary enrichment in sugar broths. Most of our water supply control laboratories are using the standard method of the American Public Health Association,

or a slight modification of it based upon initial enrichment in lactose broth, followed by isolation upon a solid medium and further confirmation when there is a question of quality of the water for drinking purposes.

Standard Methods (1925) tentatively recommends four tests for the differentiation of the fecal and non-fecal organisms. It states that "at the present time our knowledge is not sufficiently complete to warrant the adoption of any single test or group of tests and that the procedures are presented with the hope that positive evidence will gradually accumulate." Our study was made to help clarify this problem. We shall present our data in the order of the standard method procedure and attempt to show the relation of each step to the differentiation problem. The study will therefore be divided into three parts as follows: (1) Preliminary enrichment, (2) isolation, and (3) purification and differentiation. The first two parts are presented herewith.

PRELIMINARY ENRICHMENT IN LACTOSE BROTH

In the 1905 edition of *Standard Methods*, two methods of isolation of the coli-aerogenes group were suggested. Direct isolation on solid media was permissible with sewages and polluted waters containing these organisms in 1 cc. The dilution method in glucose broth was recommended for all other waters. In the 1912 edition and all later editions the dilution method only has been recommended. Today, therefore, the first step in the standard method for enumerating and isolating the coli-aerogenes group is to plant the proper portions into lactose broth. The lactose broth cultures are incubated at 37°C. and the highest dilution cultures showing gas are selected for the isolation of the organisms after 24 and 48 hours.

A review of the development of the lactose broth medium as given by Wagner and Monfort (1920), Levine (1921) and Prescott and Winslow (1924) shows that the medium was designed with the hope of obtaining the best indicator of the coli-aerogenes group. Special requirements of either section of the group were not considered. This medium is but very slightly inhibitive to non-members of the coli-aerogenes group, consequently many

other organisms, lactose fermenters and non-fermenters, multiply. This complicates the identification and differentiation of the group. Many investigators, whose studies are summarized by Janzig and Montank (1928), have worked on the causes of the so-called false presumptive tests. Greer (1928) and Norton and Barnes (1928) and others have shown that these pseudo-presumptives may be caused by the overgrowth of the coli-aerogenes group by other organisms.

The possibility of one member of the coli-aerogenes group overgrowing the other is a phase of the differentiation problem which has not been properly emphasized. Since differentiation is attempted on the culture isolated following preliminary enrichment, the relative lag periods and growth rates of the *Bact. coli* and *Bact. aerogenes* strains may determine the member that will be isolated. It is this phase of the problem that we have studied. For the sake of simplicity we have confined ourselves to the study of the simultaneous growth of *Bact. coli* and *Bact. aerogenes* in enrichment broth, although other organisms may also be present in practice.

The ferrocyanide-citrate pour-plate medium of Noble (1928) (1930) is very well adapted to this study. With this medium it is possible to follow directly the numbers of *Bact. coli* and *Bact. aerogenes* growing in a mixed culture. Direct *Bact. coli* and *Bact. aerogenes* counts with the medium, in water from Lake Michigan, have indicated that both sections of the group are often present in almost equal numbers. A study of the simultaneous growth of these organisms in lactose broth will illustrate what is happening in the routine procedure in many laboratories and may modify the interpretation of the final differential results.

A typical *Bact. coli* and a typical *Bact. aerogenes* strain were selected from our laboratory collection. When streaked on eosin-methylene-blue agar, both strains formed colonies which checked with Levine's description (1921) of the respective organisms. The *Bact. coli* strain was indol-positive, methyl-red-positive and Voges-Proskauer negative, and failed to produce visible growth in Koser's citrate solution. The *Bact. aerogenes*

strain did not produce indol, was methyl-red-negative, was Voges-Proskauer positive and utilized citrate. Numerous transplants from both of these strains during the past year have always given the same indol, methyl-red, Voges-Proskauer and citrate reactions as above. These reactions have therefore been considered constant under the treatment received in our laboratory.



FIG. 1. FERROCYANIDE CITRATE AGAR PLATE OF A *Bact. coli* AND *Bact. aerogenes* CULTURE FROM LACTOSE BROTH

Small colonies, *Bact. coli*; large colonies, *Bact. aerogenes*

These strains produced colonies on Noble's medium which were very characteristic and constant. Figure 1 shows a typical plate from a mixed culture of these organisms. The small black colonies are *Bact. coli* and the large ones are *Bact. aerogenes*. The figure illustrates that the differentiation on this medium

was very clear cut with these strains.¹ In preparing the ferrocyanide-citrate pour-plates, the latest technique described by Tonney and Noble (1930) was used.

Tryptophane broth (24 hours at 37°) cultures were used in most of these experiments. Suspensions of the pure strains were made in sterile water. Various mixtures as desired were then made with the two pure strain suspensions. The two pure strains and the various mixed suspensions were then planted in geometric dilutions into lactose broth, as are routine water samples, for enrichment. All suspensions and the lactose enrichment cultures were immediately planted into Noble's medium for the initial counts. The course of the growth of all cultures in the lactose broth at 37°C. was followed by planting from these cultures into Noble's medium after the desired intervals. All Noble's medium plates were counted after 40 to 44 hours at 37°C. Deviation in any particular in the preparation of the ferrocyanide-citrate medium or the pour-plates will result in the failure of the medium.² The care necessary in the preparation of the medium and the plates cannot be overemphasized. Experience is also required in counting correctly the two types of organisms on the plates. Although we have had considerable experience, 20 to 30 colonies were usually picked from each batch of mixed culture plates and placed in differential media to check the visual interpretation. With strains such as were used in these experiments, perfect checks were usually obtained. As an additional check on the counts of the mixed lactose broth cultures of *Bact. coli* and *Bact. aerogenes* on Noble's medium, a dilution method based on the aforementioned differential reactions of the strains was also used. Briefly, triplicate tryptophane broth tubes and triplicate Koser's citrate-solution tubes were planted in geometric dilutions, from the lactose broth mixed enrichment cultures, and after incubation, the *Bact. coli* and *Bact. aerogenes* indexes were

¹ An attempt was made to differentiate these strains on an eosin-methylene-blue pour plate, without success. In this case the smaller *Bact. coli* and *Bact. aerogenes* colonies become indistinguishable.

² Difficulties in the use of this medium are usually encountered when the method is first tried but these cannot be discussed here.

calculated from the results of the indol and citrate tests. This method proved the accuracy of the Noble's medium counts of these strains in mixed culture. The method was more cumbersome and, since it was tried largely to check the ferrocyanide citrate plate counts, it was not continued after the experiments on the first 12 mixed cultures.

RESULTS OF SIMULTANEOUS GROWTH OF *BACT. COLI* AND *BACT. AEROGENES* DURING PRELIMINARY ENRICHMENT

The growth of 35 mixtures, having various initial ratios of the two strains described, was followed in lactose broth. Of these, 10 had initial ratios of 20 or more *Bact. aerogenes* to one *Bact. coli*. Since the dilution procedure would usually eliminate the *Bact. coli* in the highest positive dilution in these cases, they need not be considered in detail. Suffice it to say that the tendency was for the ratio of *Bact. aerogenes* to *Bact. coli* to become even greater after incubation. It was found, when almost equal initial numbers of *Bact. coli* and *Bact. aerogenes* were planted in geometric dilutions, that the highest positive gas dilution sometimes contained either the coli section of the aerogenes section but not both. This may be due to the contingency of random distribution or to the failure of the last cell of one section to divide. No evidence as to the reason for this was collected and only those cases in which both *Bact. coli* and *Bact. aerogenes* grew will be considered.

Tables 1 and 2, showing the numbers of coli and aerogenes (strain 1) organisms that are obtained in standard lactose broth, indicate that in pure culture the two strains approach roughly the same population level after 24 to 48 hours. The results obtained with 25 mixtures divided according to the initial proportions of *Bact. coli* and *Bact. aerogenes* (strain 1) are shown in table 3. It is clearly shown in the mixtures that strain 1 of *Bact. aerogenes* tended to overgrow the *Bact. coli* when equal numbers of each were present at the start. When the initial planting contained from 2 to 5 *Bact. coli* for each *Bact. aerogenes*, equal numbers of both organisms were generally obtained after 24 hours in the enrichment broth. All of these experiments were

carried out with 24-hour tryptophane broth cultures. While a consideration of the work of Henrici (1928) suggests that these strains may undergo morphological and physiological changes

TABLE 1
Numbers of Bact. coli obtained in standard lactose broth at 37°C. (bacteria per cubic centimeter)

INITIAL	AFTER 24 HOURS	AFTER 48 HOURS
	<i>millions</i>	<i>millions</i>
0.20	800	1,050
11.50	290	276
1.10	130	56
0.11	100	46
210	390	
21	640	
2.1	250	
0.21	400	
171	305	365
17.1	130	715
1.7	470	455
0.17	163	665
1,280	865	1,640
185	715	1,580
19	920	1,780
3.0	560	630
0.13	675	1,530
2,550	650	340
327	465	355
32.5	280	330
2.25	610	570
0.22	690	459
0.05	720	337
0.25	378	940
0.05		768
Average.....193.4	483	708
Geometric mean....4.29	408	507

resulting in reciprocal populations (table 3) in a mixed culture, we have no data on that point at this time.

In other experiments, however, with another strain of *Bact. aerogenes* and equal numbers of *Bact. aerogenes* and *Bact. coli* present at the start, the coli consistently overgrew the aerogenes.

The results are shown in table 4. The *Bact. coli* strain used in this case was the same as that used in the previous experiments but the tryptophane culture had been in the ice box for four days. The *Bact. aerogenes* strain had the same differential reactions as the earlier one. It produced consistently smaller colonies on

TABLE 2

Numbers of Bact. aerogenes (strains 1 and 3) obtained at 37°C. in standard lactose broth (bacteria per cubic centimeter)

	INITIAL	AFTER 24 HOURS	AFTER 48 HOURS
		millions	millions
Strain 1.....	0.32	850	1,000
	0.80	500	490
	0.50	180	550
	159	710	
	15.9	520	
	1.59	250	
	192	550	970
	24	460	800
	2.0	605	960
	30	480	755
	3.0	450	1,030
	0.3	600	975
Average.....	35.8	512	837
Geometric mean.....	4.73	477	810
Strain 3.....	1,450	795	15
	158	665	71
	15.5	850	31
	2.0	1,040	338
	0.15	690	116
	0.05	530	144
Average.....	270.95	762	119.167
Geometric mean.....	6.14	745	75.5

eosin-methylene-blue agar, with less tendency to run together than the earlier *Bact. aerogenes* strain had. It also produced smaller colonies in Noble's ferrocyanide-citrate medium.

From the data in tables 3 and 4, it is evident that it is possible for either *Bact. coli* or *Bact. aerogenes* to overgrow the other in

TABLE 3

Numbers of Bact. coli and Bact. aerogenes (strain 1) obtained when grown together at 37°C. in standard lactose broth (bacteria per cubic centimeter)

PROPORTION AT START	INITIAL		AFTER 24 HOURS		AFTER 48 HOURS	
	Coli	Aero- genes	Coli	Aero- genes	Coli	Aero- genes
			millions	millions	millions	millions
More aerogenes than coli.	2	5.0	3	340		
	0.2	3.0	1	770	5.5	3,200
	8	64	2	510	1	1,050
	14	32	12	550	30	940
	12.8	25	50	600	35	730
	1.3	3	15	620	35	850
	0.13	0.25	1	635	<1	640
Average.....	5.49	18.89	12	575	17.9	1,236
Geometric mean.....	1.92	6.65	4.74	559	7.65	1,040
About equal numbers of coli and aerogenes.....	42	36	60	650	80	800
	62	16	110	330	90	900
	157	107	130	470	161	358
	15.7	10.7	160	400	125	340
	1.57	1.1			150	480
	0.16	0.11			86	196
	25	26	60	510	20	535
	2.5	2.6	75	510	30	560
	0.26	0.25	65	365	40	620
Average.....	34.02	22.19	94.2	462.1	87	532.1
Geometric mean.....	6.67	4.80	88.5	451	70.8	486
More coli than aerogenes.....	11.5	0.5	99	310	67	220
	525	80	680	460	1,450	440
	52.5	8	670	480	600	430
	5.25	0.8			680	240
	0.52	0.08			290	500
	17	3	10	320	15	275
	1.7	0.3	20	370	10	510
	0.17	0.05	1	440	10	855
	34	3	230	172	340	715
	3.4	0.3	510	1	500	115
Average.....	65.10	9.60	277.5	319.1	396.2	430
Geometric mean.....	7.4	0.98	76	170	140	370

lactose enrichment, when they are both initially present in equal numbers. In fact, the data show that it is highly probable that either one or the other is going to gain the ascendancy and greatly outnumber the other during enrichment. This probability of overgrowth may produce distorted views of the initial condition as determined by eosin-methylene-blue or other streaked isolation plates.

Before considering the possibility of distortion caused by overgrowth, we wish to discuss briefly the mechanism of the ascend-

TABLE 4

Numbers of Bact. coli and Bact. aerogenes (strain 8) obtained when grown together at 37°C. in standard lactose broth (about equal numbers of each organism at start) (bacteria per cubic centimeter)

INITIAL		AFTER 24 HOURS		AFTER 48 HOURS	
Coli	Aerogenes	Coli	Aerogenes	Coli	Aerogenes
		<i>millions</i>	<i>millions</i>	<i>millions</i>	<i>millions</i>
44	44	460	105	276	2
15	21.5	530	155	430	<10
8.5	6.0	565	100	330	15
2.5	2.0	540	120	400	10
0.23	0.24	320	160	355	30
35	25.0	475	95	415	20
12.5	8.0	455	105	435	20
3.0	4.5	510	130	570	40
2.5	1.5	400	100	515	50
2.2	0.15	170	70	380	30
Average.....	12.35	442.5	114	410.6	22.7
Geometric mean...	4.85	431	110	403	17.1

ancy of one organism over the other during their simultaneous growth in lactose broth. There seem to be three factors which enter into this ascendancy: First, the lagphase of the organisms in the medium; second, the relative maximum growth rates or, conversely, the minimum generation times of the strains in the broth; and finally, the effect of the products of metabolism of the organisms on each strain. Table 5 shows three experiments which illustrate the effect of lag on the final ascendancy of the strains. Experiment 32 shows the normal result with the first

TABLE 5
Effect of the lag phase upon the final relative populations of Bact. coli and Bact. aerogenes (strain no. 1) (bacteria per cubic centimeter)

	EXPERIMENT 32		EXPERIMENT 33		EXPERIMENT 35	
	<i>Bact. coli</i>	<i>Bact. aerogenes</i>	<i>Bact. coli</i>	<i>Bact. aerogenes</i>	<i>Bact. coli</i>	<i>Bact. aerogenes</i>
Initial.....	1.7	0.3	0.17	0.05	3.4	0.30
3.5 hours.....	23	21	<1.0	7.0	9.0	<1.0
5.5 hours.....	190	570	2.0	170	60	<10
24 hours.....	20,000,000	370,000,000	<1,000,000	440,000,000	510,000,000	1,000,000
48 hours.....	10,000,000	510,000,000	10,000,000	885,000,000	500,000,000	115,000,000

two strains used, in which both strains exhibited their more common lag phases, with the final ascendancy of *Bact. aerogenes* after 24 hours. Experiment 33 shows an unusually long lag phase for the *Bact. coli* strain, which resulted in an even greater ascendancy of the *Bact. aerogenes* after 24 hours. In experiment 26, the same strains of organisms were used on the same day, with everything identical except the number of organisms, and an unusual result was obtained. In this case, the *Bact. aerogenes* strain exhibited an unusual lag phase. This resulted in the ascendancy of the *Bact. coli* after 24 hours, which is very uncommon for these strains. This illustrates, that, with small initial numbers of two such strains, an unpredictable, abnormal lag phase in either strain may result in the ascendancy of the other strain.

During the logarithmic or maximum phase of growth, the generation times of these strains are not very different. Cohen and Clark (1919) studied the maximum growth rates of *Bact. coli* and *Bact. aerogenes* in peptone solution, with various pH adjustments. They found the average minimum generation periods for their *Bact. coli* strain over a pH range of 5.0 to 8.1 to be 24 minutes. The average minimum generation time for their *Bact. aerogenes* strain, over a pH range of 4.7 to 8.4, was 22.2 minutes. This work was done with pure cultures. Our data indicate that with *Bact. coli* and *Bact. aerogenes* the average minimum generation time is the same whether they are in pure culture or together, as long as neither of the strains in the mixture has passed the logarithmic growth phase. In our experiments with the first *Bact. aerogenes* strain, the data indicate that the average minimum generation time of the *Bact. aerogenes* was shorter than that of the coli. In our experiments with the second aerogenes strain, the reverse was true. The average generation times, in minutes, found for these coli-aerogenes mixtures and for pure cultures, in the early section of the logarithmic phase, that is, during the 2 hours following the initial $3\frac{1}{2}$ -hour incubation period, were as follows:

	NUMBER OF OBSERVATIONS	GENERATION TIME	
		Mean	Standard deviation
		<i>minutes</i>	<i>minutes</i>
<i>Bacterium aerogenes</i> No. 1.....	11	23.9	±2.2
<i>Bacterium aerogenes</i> No. 3.....	16	26.6	±2.1
<i>Bacterium coli</i>	28	24.6	±3.6

The studies showed that, except in cases where an unusual lag was encountered, the strain having the minimum generation time during the logarithmic phase gained ascendancy during the first 24 hours. In most of our experiments, the logarithmic phase was apparently maintained from 10 to 12 hours. After that the generation time gradually increased due to the products of metabolism. These products also vary in their effects on the generation times of mixed strains. The two *Bact. aerogenes* strains used in these experiments illustrate this point. The first strain could continue to multiply in the presence of the products of a considerable number of *Bact. coli*, while the second *Bact. aerogenes* strain failed to exhibit any such power.

These experiments have demonstrated the tendency of either *Bact. coli* or *Bact. aerogenes* strains to gain ascendancy in enrichment broth when both of them are present in equal numbers at the start. This may cause misleading results on streaked isolation plates in routine water analysis. On account of the factors involved, as shown in the experiments, it is impossible to predict the section which will in general gain ascendancy in lactose broth and give a faulty picture on solid isolation media. To obtain information on this point, results of the Sanitary District's survey of the Illinois river have been analyzed. In this survey, each sample is planted in triplicate portions of each dilution into Noble's medium, for a direct *Bact. coli* and *Bact. aerogenes* count. At the same time, triplicate geometric dilutions are planted into lactose broth and three eosin-methylene-blue plates are subsequently streaked. These data seem to indicate that in general the proportion of *Bact. aerogenes* increases as a result of the enrichment process. We shall not elaborate on these data at this

time, but will discuss them in detail in a later report when this special survey has been completed.

From these studies, it must be concluded that even with ideal methods of isolation and identification of the organisms present at the end of the enrichment process, the differential results following enrichment in lactose broth apparently cannot be interpreted with mathematical precision. Having studied some of the difficulties encountered in enrichment as required by the standard methods, let us examine the next step in the method, in its relation to the differentiation problem.

ISOLATION OF THE COLI-AEROGENES GROUP FOR DIFFERENTIATION

Standard Methods (1925) define the coli-aerogenes group "as including all Gram-negative non-spore-forming bacilli which ferment lactose with gas formation and grow aerobically on standard solid media." Therefore, for complete confirmation according to these methods it is necessary to show that: (1) the organism produces gas in lactose broth; (2) it will grow aerobically; (3) the aerobic colony will produce gas from lactose, and (4) the organism thus obtained is Gram-negative and a non-spore-former. Growth conditions in lactose broth have been considered in the first section of this paper. Growth of the organisms aerobically, i.e., on the surface of a solid medium is the next step in the identification of the coli-aerogenes group required by the methods of the American Public Health Association. This is usually accomplished by streaking a needle wetted with the gas-producing enrichment culture on the surface of the solid isolation medium, and examining the colonies that have developed after the proper incubation period at 37°C. It is recommended by these methods that at least two of the most typical colonies shall be picked for confirmation.

It is not stated specifically in the methods where or how the differential tests are to be applied. It is natural, therefore, for the water laboratory technician to infer that the differential tests might be applied directly to the isolated and confirmed strain.³

³ Earlier editions of *Standard Methods* (1917 and 1923) recommended that the differential tests be applied to ten coli-aerogenes group cultures from a sample.

We propose, therefore, to discuss certain problems in the isolation procedure which seem to be pertinent to the differentiation problem. The problems upon which we will present data are as follows:

1. The choice of an isolation medium.
2. The possible failure of streaked solid medium plates to isolate the organisms directly from lactose enrichment cultures.
3. The degree of purity of isolated colonies.
4. The correctness of the macroscopic differential interpretation of the colonies.
5. The possibility of a single streaked plate from a culture showing all apparently pure colonies of one type, while the parent culture and some of the colonies on the plate are actually mixed.

CHOICE OF ISOLATION MEDIUM

An ideal isolation medium may be defined as a solid medium upon which a colony would be obtained for every coli-aerogenes group organism, but on which all other types would fail to grow. An ideal medium for isolation and differentiation would have the above requisites and would also produce distinctly different colonies for the aerogenes and coli types.

A number of solid media are being used for isolating these organisms, but they are all far from ideal. Nevertheless progress has been made in the development of media with some of the above qualities.

Among the more common isolation media used are the modifications of Endo's (1904) fuchsin-lactose agar, MacConkey's (1905) neutral-red-bile-salt agar and Holt and Teague's (1916) eosin-methylene-blue agar. MacConkey's agar is used by Houston of the Metropolitan Water Board of London, but it is not used much in the United States. *Standard Methods* (1925) describes

These cultures were to be fished, preferably, from direct isolation plates but might be fished from isolation plates following preliminary enrichment. Technically it is desirable to select ten cultures per sample for differentiation. Practically, however, it is impossible for routine water laboratories to handle 10 subcultures for differentiation from each sample with the personnel usually allowed for this work.

and recommends Endo agar and Levine's modification of eosin-methylene-blue agar and these media are almost universally used.

Salle (1927) (1929) after a careful consideration of the problem of isolation and differentiation designed an erythrosin, methylene-blue, brom-cresol-purple, lactose agar. It is claimed that this medium sharply differentiates the *Bact. coli* and *Bact. aerogenes* sections on the basis of the carbohydrate metabolism of these organisms and the indicators employed, thus eliminating the methyl-red test. In pure culture this difference is no doubt true, but with mixed enrichment cultures it does not necessarily hold. A comparative study was made by us of 100 each of Salle, E. M. B. and Endo plates streaked with 100 pure and mixed cultures. The results showed that some cultures which had both *Bact. coli* and *Bact. aerogenes* type colonies on Endo and E. M. B. had only *Bact. coli*-like colonies on Salle's medium. Our brief study seemed to indicate that the medium was inferior to E. M. B. for differentiation. It may, however, be superior for isolation of the *Bact. coli* section of the group and it deserves further study by other water laboratories.⁴

Leaving Salle's medium for a more extended study we shall consider here only the Endo and eosin-methylene-blue media. Prescott and Winslow (1924) describe a parallel study of Endo agar and E. M. B. agar and conclude that it is "an open question whether eosin-methylene-blue, should or should not supersede Endo agar for isolation." Greer, Noble, *et al.* (1928) made a study of 1700 Endo and E. M. B. plates in parallel and concluded, "that the E. M. B. plates are more likely to have growth that can be confirmed as *B. coli*" (coli-aerogenes group). We have also made a parallel study of over 1,000 Endo and E. M. B. plates. Our results showed practically no difference in these media when isolation of members of the coli-aerogenes group is desired. When differentiation of the two sections of the group is an object the indications are that E. M. B. medium is superior. On the other hand the E. M. B. agar is apparently a better medium for the growth of the *Bact. aerogenes* section than Endo agar.

⁴ Preliminary studies of this medium made by Committee No. 1 of the American Water Works Association (1928) were also not favorable.

TABLE 6
Comparison of results on Levine's E. M. B. agar with Skinner and Murray's modified E. M. B., both streaked from the same positive presumptive enrichment cultures from Lake Michigan water samples

	TOTAL: 437 STREAKS		AGREEMENT ON BOTH KINDS OF PLATES: 277 STREAKS	DIFFERING RESULTS: 160 STREAKS							
	Levine's Skinner and Murray's			Levine's	Skinner and Murray's*						
					TC	TA	BC	AA	NC	Mixed	No growth
Typical coli (TC).....	53	42	31	22			15	2	1	3	1
Typical aerogenes (TA).....	44	44	31	13			2	8	1	2	
Blue coli (BC).....	36	70	19	17	5	2		4	5		1
Atypical aerogenes (AA).....	173	123	107	66	4	8	23		24	1	6
Non-characteristic growth (NC).....	73	96	54	19	1	1	6		1	2	9
Mixed growth.....	13	14	4	9		2	2	2			
No growth.....	45	48	31	14	1		3		10	2	
Totals.....	437	437	277	160	11	13	51	16	42	10	17

* For meanings of abbreviations see left-hand column of this table.

In the report of Committee No. 1 (1928), of the American Water Works Association, on standard methods of water analysis, the following recommendation is made: "that the eosin-methylene-blue agar and modifications thereof be studied to see if it is possible to obtain a more uniformly accurate differentiation between the colon and aerogenes type organisms, and that the latter be studied in regard to their tendency to overgrow organisms of the colon type." It is true that E. M. B. plates are often obtained on which a few *Bact. coli* colonies are faintly visible surrounded by masses of *Bact. aerogenes* colonies. We wish to point out that such plates may often be fairly representative of the condition which actually exists in the enrichment tube as shown in the first section of this paper. This phenomenon has, however, generally been considered overgrowth by the aerogenes section on the plates. It was apparently to eliminate this condition and the growth of spreaders on the plates that Skinner and Murray (1924) added one part of crystal violet to 100,000 parts of E. M. B. agar. Brown and Skinner (1930) reported that E. M. B. could not be used on Mississippi River water because non-lactose fermenters overgrew and prevented the isolation of coli-aerogenes organisms. This overgrowth, they report, was prevented by the use of crystal violet in the medium.

We have made a comparison of the results obtained with Lake Michigan positive presumptive cultures on Levine's E. M. B. and Skinner and Murray's modified medium. The data in table 6 show that the advantage of the crystal violet is very slight. The plates showing overgrowth by organisms other than lactose fermenters are listed as atypical aerogenes and non-characteristic growth for the coli-aerogenes group. It will be noted that while the number of plates having atypical aerogenes readings was decreased by 50 on the E. M. B. crystal violet medium, these 50 appear in the non-characteristic group and the blue coli columns. These readings are no more definite than the original atypical aerogenes readings on the E. M. B. agar. The colonies we describe as blue coli usually contain *Bact. coli*, but we find by purification and biochemical differentiation that they may contain *Bact. coli*, *Bact. aerogenes* or both (see table 7).

We conclude therefore that the addition of crystal violet to the E. M. B. agar has little advantage for the purpose of isolating or differentiating the coli-aerogenes group in the waters of the Chicago region.

We have used the Digestive Ferments Company dehydrated E. M. B. agar entirely in our studies and have found it to be quite

TABLE 7
Check of correctness of macroscopic differential interpretation of colonies on routine E. M. B. agar plates

MACROSCOPIC INTERPRETATION OF COLONIES ON E. M. B. AGAR	NUMBER OF COLONIES FISHED	DIFFERENTIAL RESULTS OF BIOCHEMICAL TESTS			PERCENTAGE OF COLONIES FISHED	
		Pure Bact. coli	Pure Bact. aero- genes	Prob- ably mixed culture*	Correct	Prob- ably mixed*
Typical <i>Bact. coli</i> colonies with metallic sheen.....	110	89	4	17	81.0	15.5
Blue coli colonies with little or no sheen.....	107	51	21	35	47.5	32.5
Coli type, large colony with sheen— resembles aerogenes.....	4	2	0	2		
Total of <i>Bact. coli</i> types.....	221	142	25	54	64.5	24.5
Typical aerogenes type.....	44	1	26	17	59.0	38.5
Aerogenes type (but not exactly typical).....	39	4	16	19	41.0	48.5
Atypical aerogenes type.....	70	3	28	39	40.0	55.5
Total of aerogenes types.....	153	8	70	75	46	49

* This group may or may not contain the organism for which the colony was fished.

uniform and satisfactory. On account of the quantities used in our laboratory fresh E. M. B. plates are poured about every other day. Any extra plates are kept in the ice box until used.

We have also undertaken a comparative productivity study of the isolation media that we have used along with Noble's ferro-cyanide-citrate agar, on pure cultures. While this study is not complete it indicates that Levine's E. M. B. (dehydrated Difco

product) seems to be more favorable to both the *Bact. coli* and *Bact. aerogenes* sections than any medium except Noble's ferro-cyanide-citrate agar. While our studies on isolation have been made on Levine's E. M. B. agar, we believe that the results obtained with it will also apply to the other solid media upon which isolation and differentiation is attempted after streaking with lactose-fermenting enrichment cultures.

FAILURE OF ISOLATION ON E. M. B. AGAR

We wish to discuss briefly the possibility of failure to isolate the organisms on solid media such as E. M. B. when the coli-aerogenes group organisms are present and viable, but are greatly in the minority in the parent culture. Usually, when no growth on E. M. B. plates is obtained from positive presumptive cultures it is assumed that the coli-aerogenes group has been overgrown by spore-forming anaerobes that have reduced the pH below the lethal point for the former group. Regardless of the cause, by referring to the lower section of table 6 it will be seen that in 14 cases, when no-growth plates were obtained on E. M. B., aerobes were isolated on the crystal-violet E. M. B. In 17 other cases when no growth was obtained on crystal-violet E. M. B., aerobes were isolated on Levine's E. M. B. It is possible that in some of the 31 cases, in which no growth was obtained on both media, aerobes might have been isolated by more perfect methods.

In one experiment 94 positive presumptive test cultures, obtained from contaminated water and sewage, were transferred simultaneously to E. M. B. and to liquid brilliant-green bile media. Only 58 isolations were obtained on the E. M. B. agar while the brilliant-green bile broth yielded 73 aerobic, lactose-fermenting cultures. All 73 proved to be either *Bact. coli* or *Bact. aerogenes* and 17 of them contained both. This was an instance where the standard E. M. B. agar yielded 15 less Coli-aerogenes isolations than another method on less than 100 presumptive tests.

EXAMINATION OF PURITY OF COLONIES ON ISOLATION MEDIA

In water examination practice it seems to be an accepted principle that isolated colonies are necessarily pure. This may be generally true, but certainly not always. A consideration of the fact that in the streaking procedure one is spreading from 100,000 to 1,000,000 or more organisms on a very small surface area (the area actually touched by the needle) shows the probability that oftentimes more than one organism is detached at the same point. If the parent cultures have almost equal numbers of two organisms that are both capable of growing on the medium and are not antibiotic, mixed isolated colonies may be expected. This often happens in routine water examination.

We have used two methods of determining the incidence of mixed growth in isolated colonies. The first method is the ordinary microscopical one and the second is the determination of the biochemical reactions of the organisms.

In the first method the picked colonies were carried through to complete confirmation including an examination of the Gram stain of the agar slant cultures. In routine work we believe that the microscopic examination for mixtures is not very dependable. Of 13,222 Gram stain examinations that were made on completely confirmed cultures in our laboratory in 1927, 1928 and 1929, only 732 or 5.5 per cent were reported as spore-forming in mixed or pure culture. In the same group 1,197 or 9.0 per cent were reported as mixed cultures without spores, while a total of 1,929, or 14.5 per cent were reported as probably mixed. From a differential biochemical examination of over 1,200 cultures that had been reported as Gram-negative, pure, non-sporeforming, in 1929, we suspected the fact that there were many more mixtures which we failed to detect microscopically. An investigation convinced us that a single examination of an agar slant culture after 24 hours failed to show all of the mixed cultures. If an examination of the slants were made after one day and three days incubation some cultures which appeared to be mixed after one day appeared to be pure after three days and vice versa. If all cultures which were considered impure at any examination were

included in the mixed class a larger proportion of mixed cultures would result. The results obtained in one such series of microscopical examinations of Gram stained smears made after the agar slants had been incubated for one day and again after one week are as follows:

ISOLATED COLI-AEROGENES GROUP COLONIES FROM E. M. B. PLATES	NUMBER OF MIXED CULTURES FOUND BY MICROSCOPICAL EXAMINATION OF AGAR SLANT CULTURES		
	After one day	After one week	Total mixtures on either examination
Numbers 539.....	157	96	210
Per cent 100.....	29.3	17.8	39.8

These figures are not strictly accurate for it is possible that some smears which were reported as mixtures were not true mixtures, but contained sufficient numbers of involution forms so that they were mistaken for mixtures. On the other hand the cells of *Bact. coli* and *Bact. aerogenes* type organisms are so nearly alike that it is impossible to recognize a mixture of this character under the microscope. To determine this point, Gram stains of smears of various pure *Bact. coli* and *Bact. aerogenes* strains and coli-aerogenes experimental mixtures were examined and none of the observers were able to differentiate any of them by simple microscopic inspection of the stained smear. Consequently mixtures of pure *Bact. coli* and *Bact. aerogenes* were missed in the examination of these cultures. The source of the water, the season of the year, the technique of streaking and picking colonies, the agar slant incubation periods, the number of smears examined after various incubation times all affect the number of mixtures found by this method. Nevertheless these data show that a considerable portion of the isolated colonies picked from routine plates which might ordinarily be considered pure are not pure. We also conclude that the microscopical examination of coli-aerogenes group cultures as a test for purity is deficient and not satisfactory.

The biochemical method of detecting mixed colonies has the advantage that it practically always indicates coli-aerogenes

mixtures. This method will be described in greater detail later. It has the disadvantage, however, that it does not always indicate mixtures of either section of the coli-aerogenes group with certain other organisms. Also a small portion of the cultures which might be considered mixed by these biochemical tests may actually be found to be unusual strains. Using this method we picked 374 single isolated colonies from routine E. M. B. plates of Lake Michigan water cultures. The indications were that 245 of these were pure members of the coli-aerogenes group. The remaining 129 were mixtures of *Bact. coli* with *Bact. aerogenes*, *Bact. coli* or *Bact. aerogenes* with other organisms not belonging to the group and a very few may have been unusual coli-aerogenes forms. In other words this method indicated that about 35 per cent of these isolated colonies which were selected as belonging to the coli-aerogenes group were probably mixtures.

DIFFERENTIATION OF COLONIES BY MACROSCOPIC APPEARANCE ON ISOLATION MEDIA

The above experiments showed that isolated colonies picked from plates streaked with enrichment cultures cannot be depended upon as being pure. It may be contended by some that even though such colonies are not pure their appearance will indicate to which section of the coli-aerogenes group they belong. Levine (1921) reported that 96.9 per cent of 102 colonies fished as *Bact. coli* proved correct while 82.4 per cent of 122 colonies fished as *Bact. aerogenes* proved correct. Georgia and Morales (1926) reported that in general they were able to differentiate the *Bact. coli* and *Bact. aerogenes* colonies on E. M. B. plates from water samples. They also report, however, that in a number of instances colonies that were picked for one type proved to be the other. Our results more closely approach the finding of the latter workers. Table 7 shows the results obtained with the 374 colonies mentioned above. The data show that in general the percentage correctness of the macroscopic interpretation decreases as the colonies become less typical in appearance. Nevertheless a great many routine plates contain no typical colonies but contain members of the coli-aerogenes group, as the

atypical aerogenes data in this table show. It also appears from these data that the proportion of mixed culture colonies is greater for the aerogenes type than for the coli type. The difference in the carbohydrate metabolism of the two types may partly explain this. If it be assumed that each of the mixed colonies found also contains the organisms for which the colony was fished, the correctness of the macroscopic differentiation would be very satisfactory. Unfortunately, however, further purification proves that this is not the case.

The cultures obtained from colonies such as are classified in the mixed group in table 7 were purified very carefully. They were cultured in lactose broth and restreaked on E. M. B. from 5 to 7 times, each time colonies having any variations in appearance being picked so that as few as possible might be lost.

Of 61 such colony cultures pure *Bact. coli* and pure *Bact. aerogenes* were isolated in each of 18 colonies thus picked. In a few cases a non-lactose fermenting organism was isolated with the coli-aerogenes group organism. In 7 cases anomalous coli-aerogenes group organisms were finally obtained. In 8 cases the coli-aerogenes forms were lost and only non-lactose fermenters were isolated. In the remainder of the cases either pure *Bact. coli* or pure *Bact. aerogenes* were obtained, but the organisms that had been mixed with them at the start had been lost. It may be well to point out here that these data on purification serve to illustrate the uncertainties of purifying a culture by repeated cultivation in lactose broth and restreaking on E. M. B. agar. The first section of this paper illustrates the danger of losing one member of a mixture by this process. In this experiment both *Bact. coli* and *Bact. aerogenes* organisms were isolated from single isolated typical *Bact. aerogenes* colonies. Our work leads us to believe that a macroscopical interpretation of the first streaked isolation plate is very unscientific and is in many cases pure conjecture. Combinations of organisms produce colonies which are very misleading. Our atypical aerogenes on E. M. B. agar plates for instance often consist of some member of the coli-aerogenes group overgrown with other organisms. For instance 1519, or 57 per cent of 2,646 routine plates from various Lake

Michigan water intakes and filter plants, contained only such atypical colonies. Table 7 shows that they must be repurified before they can even be differentiated by biochemical tests, to say nothing of macroscopic interpretation.

One of the organisms which apparently causes much of this difficulty is a non-lactose fermenting, non-spore forming, Gram-negative rod. This organism corresponds closely to one isolated from soil by Koser and Shinn (1927) and utilizes citrate. It also causes difficulty as one of the so-called "extraneous" forms that is capable of growing on Noble's ferro-cyanide citrate medium. With this medium we have found that it often outnumbers the coli-aerogenes group 10 to 1 in Lake Michigan water. Although we have not as yet investigated its growth rate in lactose broth we have every reason to believe that it often outgrows the coli-aerogenes group. Fortunately it does not produce acid in lactose broth and does not prevent the increase of the coli-aerogenes group. However, these organisms grow luxuriantly on E. M. B. agar, producing pale, flat colonies in such large numbers that they often seem to overgrow all other forms. When the enrichment culture contains many more of these organisms than *Bact. coli*, these organisms apparently mask the colony appearance of *Bact. coli*. Such growth often results in what we interpret as atypical aerogenes colonies, for there is no indication of *Bact. coli* on the plate. Further careful purification and differentiation, however, result in the separation of *Bact. coli* and these non-lactose fermenting soil forms.

Another group of organisms which causes trouble during the isolation of routine cultures is the *Clostridium Welchii* group. We have studied these organisms both in pure and mixed cultures with the coli-aerogenes group. They have also been studied when they were isolated from single colonies on routine plates and purified, and from experimentally mixed cultures streaked on plates. Our tentative conclusions were that pure cultures of these organisms rarely if ever grew on Endo, E. M. B. or Salle's agar. With coli-aerogenes cultures and possibly other aerobes they grew on all of these media. Growing with coli-aerogenes strains they restricted the size and masked the appearance of

the colonies formed. The colonies produced are pin point sheen colonies (sometimes slightly larger) and are usually considered as non-members of the coli-aerogenes group. They may be confused with colonies of *B. aerosporus* and also *Streptococcus fecalis*. We have isolated the spore-forming section of these routine colonies by pasteurization of subcultures and obtained cultures which failed to grow on E. M. B. but fermented lactose broth. It is much more difficult to purify these cultures and isolate the coli-aerogenes group, but in several instances we succeeded in isolating typical aerogenes strains from such colonies. This discussion of mixed colony possibilities and the masking effect of extraneous forms indicates further the difficulties of macroscopic interpretation of routine streaked plates.

The correctness of the macroscopic examination of the coli-aerogenes type colonies for purity when the plates were streaked with mixed suspensions of pure *Bact. coli* and pure *Bact. aerogenes* was also determined. Fifteen pure *Bact. coli* and 15 pure *Bact. aerogenes* strains, all of which formed colonies typical of their type and easily distinguishable from the other types on E. M. B. agar plates, were selected, as well as one *Bact. coli* strain, No. 207, and one *Bact. aerogenes* strain, No. 211, from the American Type Culture Collection. All of these *Bact. coli* strains had the same biochemical differential characteristics described for the *Bact. coli* used in the first section of this paper. In the same way all *Bact. aerogenes* strains checked the reactions of the *Bact. aerogenes* in the preliminary enrichment section. The purity and type of the single isolated colony were, therefore, easily checked by fishing into tryptophane broth and Koser's citrate solution and making the indol and visible citrate growth tests. In this experiment 71 plates of these experimentally mixed *Bact. coli* and *Bact. aerogenes* cultures were streaked and 606 colonies were fished from them. These have been grouped according to the plate characteristics as shown on following page.

CLASS	DESCRIPTION OF STREACHED PLATES	NUMBER OF PLATES EXAMINED	TOTAL COLONIES FISHED	COLONIES ON WHICH MACROSCOPIC INTERPRETATION WAS INCORRECT	
				Number	Per cent
1	About equal numbers of well-isolated colonies of both types	53	444	117*	26.3
2	Aerogenes greatly outnumbered coli in parent culture. Well isolated colonies, but plates contain 10 or more aerogenes colonies for each coli colony	11	118	25*	21.2
3	Both types present but colonies not well isolated. Some adjacent colonies picked	4	19	14†	73.0
4	No visible evidence of <i>Bact. coli</i> colonies. Colonies all apparently <i>Bact. aerogenes</i>	3	25	6†	24.0

* These were either mixed or were pure but when pure they did not contain the type for which they were fished.

† These were all mixed colonies containing both types.

The data obtained with class 1 and 2 plates have been assembled in table 8 for clarity. This experiment supports the evidence previously presented in regard to mixed isolated colonies and incorrect macroscopic interpretation on routine plates. It enables us to draw these additional conclusions: First, that the incidence of isolated impure colonies is apparently lower with pure *Bact. coli* and pure *Bact. aerogenes* mixtures than when these organisms are mixed with other non-members of the group. Second, that the macroscopic differential interpretations of the type is easier and more reliable if non-members of the coli-aerogenes group have been eliminated. Third, that the incidence of mixed colony formation seems to vary with different combinations of *Bact. coli* and *Bact. aerogenes* strains.

MULTIPLE PLATE METHOD OF DETERMINING MIXED CULTURES

So far we have shown that typical *Bact. coli* colonies may contain both *Bact. coli* and *Bact. aerogenes*. In class 4 plates of the experiment just described we found that plates could have

only typical *Bact. aerogenes* colonies on them and still contain *Bact. coli*. We have shown these phenomena only by biochemical differential tests. Therefore the validity of the biochemical differential test method for purity has been checked with a multiple streaked plate method. At the same time it was illustrated that a single streaked plate does not give the complete picture of the parent culture.

TABLE 8

Study of purity of isolated colonies on E. M. B. plates streaked from laboratory mixed cultures of Bact. coli and Bact. aerogenes having different biochemical differential reactions

CLASS OF PLATE	TYPE OF COLONY FISHED	TOTAL ISO-LATED COLONIES PICKED	TOTAL PURE COLONIES IRRESPECTIVE OF TYPE INTERPRETATION	TOTAL COLONIES CONTAINING BOTH TYPES	TOTAL PURE COLONIES IN WHICH INTERPRETATION OF TYPE WAS INCORRECT	TOTAL COLONIES IN WHICH INSPECTION DIAGNOSIS WAS INCORRECT	PERCENTAGE OF TOTAL COLONIES PICKED		
							Pure colonies irrespective as to type interpretation	Colonies containing both types	Colonies in which inspection diagnosis was incorrect
A	Aero-genes	279	232	47	25	72	83	17	26
	Coli	165	125	40	5	45	76	24	27
Total A.....		444	357	87	30	117	80.4	19.6	26.3
B	Aero-genes	97	85	12	3	15	87.5	12.5	15.5
	Coli	21	11	10	0	10	52.5	47.5	47.5
Total B.....		118	96	22	3	25	81.5	18.5	18.6
Grand total..		562	453	109	33	142	80.5	19.5	25.3

In this experiment 119 strains from routine work were used after they had been completely confirmed by the standard method and had been reported as Gram-negative, pure and non-spore-forming after one Gram stain examination. These strains were fished from agar slant cultures into tryptophane broth and incubated 24 hours at 37°C. Transfers with a loop were then made into standard lactose broth, three lactose broths with different amounts of buffer salt and into four brilliant-green-lactose-pep-

TABLE 3
Determination of mixed cultures by the multiple plate method and the biochemical differential method

CLASSIFICATION	SINGLE E. M. B. PLATE STREAKED FROM LACTOSE BROTH		EIGHT E. M. B. PLATES STREAKED FROM 8 TUBOUS LACTOSE BROTHS		BIOCHEMICAL DIFFERENTIAL TESTS		PERCENTAGE RESULTS		
	Classification based on one E. M. B. plate	Number	Classification based on eight E. M. B. plates	Number	Classification based on differential tests	Number	One E. M. B. plate	Eight E. M. B. plates	Differential tests
Pure <i>Bact. coli</i>	Plate contained only <i>Bact. coli</i> type	59	All plates showed only <i>Bact. coli</i> types	30	Regular <i>Bact. coli</i> reactions	29	50.5	25.5	24.4
Pure <i>Bact. aerogenes</i>	Plate contained only typical <i>Bact. aerogenes</i> colonies	20	All plates showed only typical <i>Bact. aerogenes</i> colonies	11	Regular <i>Bact. aerogenes</i> reactions	9	17	9.5	7.6
Doubtful	Only one type colony but atypical of either section	22	Only one type colony on all plates but interpretations did not check	55	Irregular reactions indicating mixed or anomalous strains	81	32.5	65.0	68.0
Mixed	Two types of colonies found on the plate	16	Two types found on one or more of the 8 plates	21					

tone-bile broths, three of which contained buffer and one of which did not. The media have been described by Ruchhoft, Kallas and Chinn (1930). At the same time the cultures were transferred for the biochemical differential tests. All of the fermentation media were incubated for 48 hours at 37°C. and then each culture from each medium was streaked on E. M. B. agar. After 24 hours at 37°C. the series of E. M. B. agar plates from each medium was examined macroscopically and interpreted independently, i.e. without reference to the results of that same culture on any of the plates from other media. Some weeks later, when the possibility of using these data to check the differential results was realized, they were assembled in table 9. The table clearly shows that the readings of the single E. M. B. plate fail to check the differential results. On the other hand, the multiple plate readings from various broths show that many of the cultures that were apparently pure on one plate were in reality not pure. At the same time the multiple plate results check the differential results remarkably well.

Let us briefly consider the apparent mechanism of this multiple plate method. We have shown earlier in this paper that our *Bact. aerogenes* strain No. 1 usually outgrew the *Bact. coli* used in the experiment in lactose broth. At the same time this pair of strains were grown together in the same way in brilliant green bile broth with reciprocal results, i.e. the *Bact. coli* outgrew the *Bact. aerogenes*. It has also been shown by Ruchhoft, Kallas and Chinn (1930) that in respect to lactose broth the brilliant-green-bile medium has little effect on the gas production of the *Bact. coli* section. On the other hand it reduces the gas produced by the *Bact. aerogenes* section, particularly during the first 24 hours. It is the relative growth rates of the coli-aerogenes group organisms and other organisms in these media that explain the results obtained with mixed cultures as shown on streaked plates according to *Standard Methods*. The above multiple plate method is crude (for it was not designed for the purpose for which it was used) and we believe that a much better one could be found. Nevertheless it shows again that the Gram stain examination and one streaked plate on solid media may give a

false indication of the purity of a culture. Furthermore, the experiment shows the validity of the differential method, which will be described in the next section, as a test for purity.

SUMMARY

We may summarize our study on isolation of the coli-aerogenes group for differentiation as follows:

1. That Levine's E. M. B. or Skinner and Murray's modification of it seems to be the best for isolation, but that neither of these is ideal.

2. Though the single streaked plate from a positive presumptive culture contains only one type of apparently pure colonies, the mother culture and even the colonies on the plate may be mixed.

3. That many isolated colonies that are usually considered pure are not pure and that organisms not belonging to the group sometimes mask the colonial characteristics of the coli-aerogenes group. When two or more distinct types of colonies appear on a plate the isolated colonies of either type may be contaminated with the other.

4. Due to the incidence of mixed colonies and the masking effect of the non-members of the group, differentiation on the routine E. M. B. plate by macroscopic examination is not successful.

The above conclusions were reached after studying isolated colonies from solid medium plates streaked with routine and experimentally mixed cultures, using the following three methods which supported each other: (a) microscopical examination of stained smears; (b) biochemical differential reactions; (c) multiple media, multiple plate method.

We may add finally, that biochemical differential results of mixed cultures cannot be correctly interpreted and therefore, even though *Standard Methods* does not mention purification, that is the next step required for differentiation of the coli-aerogenes group.

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INDEX TO VOLUME XXI

Agglutination, The, titre of milk as an indicator of the presence of milk as an indicator of the presence of <i>Bact. abortus</i> in milk. Abstract.....	53
Ammonia and indol production, The effect of yeast on, by bacteria in culture and in feces suspension.....	225
Anaerobe, Cellulose-fermenting, Isolation and study of an apparently wide-spread, <i>Cl. cellulosolvens</i> (N. sp.?).....	167
Ascobacillus, Conjunction of disexual forms in. A preliminary report.....	305
Atwood, Catharine, Medalia, Leon S. and Bailey, Karl R. A modified Loeffler's blood serum medium useful in the routine health department examination for diphtheria and streptococcus infections.....	119
<i>B. actinoides</i> variety <i>muris</i> , The biological characters of.....	183
<i>Bacillus diphtheriae</i> in its relationship to bacteriophage.....	75
— <i>radiobacter</i> in reference to commercial legume inoculants. Abstract..	35
Bacteria, Fate of fresh water, in the sea.....	287
— producing trimethylene glycol. Abstract.....	6
—, Studies on the influence of, on the oxidation-reduction potential of milk.	
I. Influence of pure cultures of milk organisms.....	239
—, Studies on the influence of, on the oxidation-reduction potential of milk.	
II. Influence of associated cultures of milk organisms.....	253
—, Studies on the proteolytic, of milk. V. Action of proteolytic bacteria on milk serum.....	263
—, The effect of sterilization of media upon their growth promoting properties toward.....	299
—, The effect of yeast on ammonia and indol production by, in culture and in feces suspension.....	225
—, The staining of flagella of, with special reference to mordants.....	313
Bacterial growth-inhibiting substance, Do broth culture filtrates contain a?.....	395
Bacterial media, solid, A comparison of colorimetric and potentiometric methods for hydrogen ion determination of, using a dilution method based on the buffer equation.....	383
— spores.....	330
— spores, Influence of surface tension upon the germination of.....	211
—, The, culture as an electrical half-cell. Abstract.....	18
Bactericidal, The, efficiency of certain organic peroxides. Abstract.....	55
Bacteriological, A, incubator for uniform temperatures with accurate control. Abstract.....	24
— studies before and after chlorination at the Madison sewage disposal plant. Abstract.....	9
—, The, evaluation of some new water soluble organomercury compounds..	323

Bacteriology and medicine, Reciprocal effects of the relationship of.....	61
—, The, of household dusts. Abstract.....	14
Bacteriophage, A, for <i>B. anthracis</i>	161
—, A quantitative study of the respiration of staphylococcus cultures lysed by.....	143
—, <i>Bacillus diphtheriae</i> in its relationship to.....	75
Bailey, Karl R., Medalia, Leon S. and Atwood, Catharine. A modified Loeffler's blood serum medium useful in the routine health department examination for diphtheria and streptococcus infections.....	119
Baird, Lenna A. and Burke, Victor. Fate of fresh water bacteria in the sea...	287
Baldwin, I. L., Eckhardt, Marie McL. and Fred, E. B. Studies of the root-nodule organism of <i>Lupinus</i>	273
Barnes, L. A. Do broth culture filtrates contain a bacterial growth-inhibiting substance? Abstract.....	10
Barnes, L. A. Do broth culture filtrates contain a bacterial growth-inhibiting substance?.....	305
Bates, P. K. and Prescott, Samuel C. On the relation of refrigeration temperatures to the rate of growth of certain specific types of bacteria causing food spoilage. Abstract.....	25
Bates, Philip K. and Prescott, Samuel C. The reduction of the number of organisms in water as a result of freezing in domestic refrigerators. Abstract.....	26
—, —, —, — and Needle, H. C. The effect of discontinuous refrigeration on bacteria in foods. Abstract.....	25
Bayne-Jones, Stanhope. Reciprocal effects of the relationship of bacteriology and medicine.....	61
Behrens, C. A. Multiple antibody production. Abstract.....	46
Bile as an antigen in serum diagnosis of syphilis. Abstract.....	48
Biochemical studies of bacterial derivatives. XV. Skin reactions in man: A comparison of tuberculin O.T., human tubercle bacillus protein MA 100 and timothy bacillus protein MA 100. Abstract.....	57
Biological and chemical studies of the <i>Lactobacillus</i> genus with special reference to xylose fermentation. Abstract.....	4
— studies of the acid-fast organism. Abstract.....	11
—, The, characters of <i>B. actinoides</i> variety <i>mauris</i>	183
Boak, R. A. and Carpenter, C. M. Lethal temperatures for <i>Br. abortus</i> with special reference to pasteurization. Abstract.....	54
Broadhurst, Jean, Moriyama, Yone, and Pease, Dorothy. Conjunction of dissexual forms in <i>Ascobacillus</i> . A preliminary report.....	305
—, and Pease, Dorothy. Dimorphic sexual reproduction in bacteria. Abstract.....	9
Broth culture filtrates, Do, contain a bacterial growth-inhibiting substance?..	395
Brunstetter, B. C. and Magoon, C. A. Studies upon bacterial spores. III. A contribution to the physiology of spore production in <i>B. mycoides</i> . Abstract.....	33
Buffer equation, A comparison of colorimetric and potentiometric methods for hydrogen ion determination of solid bacterial media, using a dilution based on the.....	383

Burke, Victor and Baird, Lenna A.	Fate of fresh water bacteria in the sea. . .	287
—, Conn, H. J., Chairman, Cohen, Barnett, Genung, Elizabeth F., Hall, Ivan C., Kulp, W. L.	Report of committee on bacteriological technic: Progress during 1930. Abstract.	1
Burkey, L. A.	Methods for determining the persistence of certain important bacteria in Swiss cheese. Abstract.	40
Burrows, William and Tanner, F. W.	The viability of yeasts in sucrose solu- tions. Abstracts.	32
Cameron, E. J.	The "natural" or "normal" resistance of spores of thermo- philic bacteria. Abstract.	31
Carbohydrate utilization, End products of, as determined in synthetic media cultures. Studies on carbon metabolism of organisms of the genus <i>Mycobacterium</i> . III.		375
Carbon metabolism of organisms of the genus <i>Mycobacterium</i> , Studies on. II. Utilization of organic compounds in a synthetic medium.		361
— — — — —, Studies on. III. End products of carbohydrate utilization as determined in synthetic media cultures.		375
Carpenter, C. M. and Boak, R. A.	Lethal temperatures for <i>Br. abortus</i> with special reference to pasteurization. Abstract.	54
Casman, E. P. and Rettger, L. F.	Some factors limiting bacterial growth at higher temperatures. Abstract.	27
Castaneda, M. Ruiz and Zinsser, Hans.	Studies on typhus fever. Abstract..	43
Characteristics, The, of certain microorganisms as modified by the reaction of the medium. Abstract.		18
Chinn, Ben, Ruchhoff, C. C., Kallas, J. G. and Coulter, E. W.	<i>Coli-aerogenes</i> differentiation in water analysis.	407
<i>Cl. cellulovens</i> (N. sp.?), Isolation and study of an apparently widespread cellulose-fermenting anaerobe.		167
Clague, J. A. and Fellers, C. R.	Time-temperature-humidity relations in dried fruit pasteurization. Abstract.	30
Clark, L. T. and Ferry, N. S.	Studies of the properties of a bouillon filtrate of the gonococcus. Abstract.	58
Clifton, C. E.	The problem of ultrafiltration. Abstract.	7
Cohen, Barnett.	The bacterial culture as an electrical half-cell. Abstract..	18
—, Conn, H. J., Chairman, Burke, Victor, Genung, Elizabeth F., Hall, Ivan C., Kulp, W. L.	Report of committee on bacteriological technic: Progress during 1930. Abstract.	1
<i>Coli-aerogenes</i> differentiation in water analysis.		407
Colonial variation of <i>Escherichia coli</i> on solid media with especial reference to the production of rough colonies. Abstract.		13
Colorimetric and potentiometric methods, A comparison of, for hydrogen ion determination of solid bacterial media, using a dilution method based on the buffer equation.		383
Colosi, Natale.	Bile as an antigen in serum diagnosis of syphilis. Abstract..	48
Comparative, A, and taxonomic study of thirty-eight strains of <i>Bacillus ter-</i> <i>tius</i> (Henry). Abstract.		2
— investigations on the microbiological composition and the quality of butter. Abstract.		41

Comparison, A, of colorimetric and potentiometric methods for hydrogen ion determination of solid bacterial media, using a dilution method based on the buffer equation.....	383
—, A, of methods for determination of potency as observed on a group of horses immunized against diphtheria. Abstract.....	46
Conjunction of disexual forms in <i>Ascobacillus</i> . A preliminary report.....	305
Conn, H. J. The effect of certain simple non-nitrogenous salts on the growth of bacteria in soil. Abstract.....	35
—, Chairman, Burke, Victor, Cohen, Barnett, Genung, Elizabeth F., Hall, Ivan C., Kulp, W. L. Report of committee on bacteriological technic: Progress during 1930. Abstract.....	1
Continuous, The, lactic fermentation of whey. Abstract.....	37
Contribution, A, to the physiology of spore production in <i>B. mycoides</i> . Studies upon bacterial spores. III. Abstract.....	33
Copper sulphate medium for the isolation of <i>Br. abortus</i> (Bang) from milk. Abstract.....	53
Coulter, E. W., Ruchhoft, C. C., Kallas, J. G. and Chinn, Ben. Coli-aerogenes differentiation in water analysis.....	407
Cowles, Philip B. A bacteriophage for <i>B. anthracis</i>	161
— and Rettger, Leo F. Isolation and study of an apparently widespread cellulose-fermenting anaerobe, <i>Cl. cellulosolvens</i> (N. sp.?).....	167
Cromwell, H. W. and Moore, Marjorie B. Studies on pollen and pollen extracts. VI. Rabbit skin reactions in quantitative estimation of activity of pollen extracts. Abstract.....	59
Cultural characteristics of <i>Neisseria gonorrhoeae</i> . Abstract.....	48
Curran, Harold R. Influence of osmotic pressure upon spore germination....	197
— Influence of surface tension upon the germination of bacterial spores....	211
— The origin of the growth restraining substance in raw milk and the action of this substance upon an organism of the sweet curdling type. Abstract.....	38
Delves, Edna, Hadley, Philip, Klimek, John. The filtrable forms of bacteria. Abstract.....	7
Demeter, Karl J. and Maier, F. X. Comparative investigations on the microbiological composition and the quality of butter. Abstract.....	41
Demonstration of a simple anaerobic culture dish. Abstract.....	23
Denitrifying organism, A marine.....	89
Development of flagella on germinating spores.....	357
Dimorphic sexual reproduction in bacteria. Abstract.....	9
Diphtheria and streptococcus infections, A modified Loeffler's blood serum medium useful in the routine health department examination for.....	119
Direct, A, method for the determination of the H-ion concentration of cultures. Abstract.....	22
Disexual forms, Conjunction of, in <i>Ascobacillus</i> . A preliminary report....	305
Dissociation in an encapsulated staphylococcus.....	157
— of <i>Myc. leprae</i> . Abstract.....	12
Do broth culture filtrates contain a bacterial growth inhibiting substance? Abstract.....	10

Do broth culture filtrates contain a bacterial growth inhibiting substance?...	395
Domogalla, Bernard. Bacteriological studies before and after chlorination at the Madison sewage disposal plant. Abstract.....	9
Downs, Cornelia M. Studies of tularemia infection in rabbits and guinea pigs. Abstract.....	50
— and Gottlieb, Selma. Studies on the precipitin reaction. III. Further studies on conditions influencing the formation of precipitates. Abstract.....	47
Drying, The, of bacteria and the viability of dry bacterial cells. Abstract....	13
Eaton, Monroe D., Jr. A quantitative study of the respiration of staphylococcus cultures lysed by bacteriophage.....	143
Eckhardt, Marie McL., Baldwin, I. L. and Fred, E. B. Studies of the root-nodule organism of <i>Lupinus</i>	273
Effect, The, of bacterial by-products on leucocytes. Abstract.....	42
—, The, of certain simple non-nitrogenous salts on the growth of bacteria in soil. Abstract.....	35
—, The, of concentrated salt and sugar solutions on the thermo-death times of molds. Abstract.....	32
—, The, of discontinuous refrigeration on bacteria in foods. Abstract.....	25
—, The, of sterilization of media upon their growth promoting properties toward bacteria.....	299
—, The, of temperature upon the production of hydrogen sulphide by <i>Salmonella pullorum</i>	111
—, The, of urine on hemolytic streptococci. Abstract.....	49
—, The, of yeast on ammonia and indol production by bacteria in cultural and in feces suspension.....	225
Effects, The, of alkaloids on bacteria. Abstract.....	55
Electrophoresis studies on <i>Lactobacillus acidophilus</i> and <i>Lactobacillus bulgaricus</i> cultures. Abstract.....	51
End products of carbohydrate utilization as determined in synthetic media cultures. Studies on carbon metabolism of organisms of the genus <i>Mycobacterium</i> . III.....	375
Ergosterol content of certain species of <i>Mycobacterium</i> and the effect of ergosterol (activated and unactivated) on their growth. (final report) Abstract.....	9
Evans, Alice C. The effect of bacterial by-products on leucocytes. Abstract.....	42
Factors, Some, limiting bacterial growth at higher temperatures. Abstract..	27
Fate of fresh water bacteria in the sea.....	287
Feces suspension, The effect of yeast on ammonia and indol production by bacteria in culture and in.....	225
Fellers, C. R. and Clague, J. A. Time-temperature-humidity relations in dried fruit pasteurization. Abstract.....	30
Fermentation, The, of polyhydric alcohols by the colon-aerogenes groups of bacteria. Abstract.....	11
Ferry, N. S. and Clark, L. T. Studies of the properties of a bouillon filtrate of the gonococcus. Abstract.....	58

Field, J. Thomas and Poe, Charles F. The fermentation of polyhydric alcohols by the colon-aerogenes groups of bacteria. Abstract.....	11
Filtrable, The, forms of bacteria. Abstract.....	7
Flagella, Development of, on germinating spores.....	357
— of bacteria, The staining of, with special reference to mordants.....	313
Floral changes in the fermentation of sauerkraut. Abstract.....	36
Foter, M. J. and Stark, C. N. The importance of feeds as a source of ropy milk organisms. Abstract.....	37
Fowls and chickens, Saprophytic and secondary microorganisms occurring in the respiratory tracts of domestic fowls and chickens in health and disease.....	97
Frazier, William C. and Rupp, Philip. Studies on the proteolytic bacteria of milk. V. Action of proteolytic bacteria on milk serum.....	263
— and Whittier, Earle O. Studies on the influence of bacteria on the oxidation-reduction potential of milk. I. Influence of pure cultures of milk organisms.....	239
— and —. Studies on the influence of bacteria on the oxidation-reduction potential of milk. II. Influence of associated cultures of milk organisms.....	253
— and Wing, Helen U. Studies on the eye-forming organism of Swiss cheese. Abstract.....	39
Fred, E. B., Eckhardt, Marie McL. and Baldwin, I. L. Studies of the root-nodule organism of <i>Lupinus</i>	273
Fulmer, Ellis I., Williams, Arthur L. and Werkman, C. H. The effect of sterilization of media upon their growth promoting properties toward bacteria.....	299
Funk, E. H., Huntoon, F. M. and White, Harry. Biochemical studies of bacterial derivatives. XV. Skin reactions in man: A comparison of tuberculin O.T., human tubercle bacillus protein MA 100 and timothy bacillus protein MA 100. Abstract.....	57
Further studies on conditions influencing the formation of precipitates. Studies on the precipitin reaction. III. Abstract.....	47
Genung, Elizabeth F., Conn, H. J., Chairman, Burke, Victor, Cohen, Barnett, Hall, Ivan C., Kulp, W. L. Report of committee on bacteriological technic: Progress during 1930. Abstract.....	1
Genus, The, leuconostoc. Abstract.....	3
Germinating spores, Development of flagella on.....	357
Gibbs, Charles S. Do saprophytic and secondary bacteria occurring in the respiratory tracts of domestic fowl in infectious trachitis and in health produce agglutinins in the blood? Abstract.....	45
— Saprophytic and secondary microorganisms occurring in the respiratory tracts of domestic fowls and chickens in health and disease.....	97
Gilbert, Isabelle. Dissociation in an encapsulated staphylococcus.....	157
Gillen, G. and Werkman, C. H. Bacteria producing trimethylene glycol. Abstract.....	6
Gilman, Herbert L. The agglutination titre of milk as an indicator of the presence of <i>Bact. abortus</i> in milk. Abstract.....	53

Gottlieb, Selma and Downs, Cornelia M. Studies on the precipitin reaction. III. Further studies on conditions influencing the formation of precipitates. Abstract.....	47
Gradwohl, R. B. H. Cultural characteristics of <i>Neisseria gonorrhoeae</i> . Abstract.....	48
Hadley, Philip, Delves, Edna, and Klimek, John. The filtrable forms of bacteria. Abstract.....	7
Hale, J. F., Prescott, Samuel C., and White, G. E. Observations on the bacteriology of slimy beef. Abstract.....	26
Hall, Ivan C., Conn, H. J., Chairman, Burke, Victor, Cohen, Barnett, Genung, Elizabeth F., and Kulp, W. L. Report of committee on bacteriological technic: Progress during 1930. Abstract.....	1
— and VanVranken, Marjorie. A comparative and taxonomic study of thirty-eight strains of <i>Bacillus tertius</i> (Henry). Abstract.....	2
Health department examination for diphtheria and streptococcus infections, A modified Loeffler's blood serum medium useful in the routine.....	119
Hemolytic streptococcus carriers, An improved method for the detection of.....	219
Herrington, B. L. and Stark, C. N. The drying of bacteria and the viability of dry bacterial cells. Abstract.....	13
Hicks, Robert Alan and Szymanowski, Wacław T. A study of the action of short electric waves on several biological substances with special reference to diphtheria toxin. Abstract.....	59
Hollon, Harriett C. and Kendrick, Pearl. Serologic and bacteriophagic relationships in a group of fecal streptococci. Abstract.....	49
Horwood, Murray P. The bacteriology of household dusts. Abstract....	14
Hotchkiss, Margaret. Presence of fusiform bacilli and spirochetes in the mouths of a group of young adults. Abstract.....	44
Hucker, G. J. and Pederson, C. S. The genus <i>leuconostoc</i> . Abstract.....	3
Huddleson, I. Forest. Copper sulphate medium for the isolation of <i>Br. abortus</i> (Bang) from milk. Abstract.....	53
Humus as a source of energy for soil microorganisms, and the energy liberated by their activity. Abstract.....	33
Hunter, Charles A. Colonial variation of <i>Escherichia coli</i> on solid media with especial reference to the production of rough colonies. Abstract.....	13
Huntoon, F. M., Funk, E. H. and White, Harry. Biochemical studies of bacterial derivatives. XV. Skin reactions in man: A comparison of tuberculin O.T., human tubercle bacillus protein MA 100 and timothy bacillus protein MA 100. Abstract.....	57
Hydrogen ion determination of solid bacterial media, A comparison of colorimetric and potentiometric methods for, using a dilution method based on the buffer equation.....	383
— sulphide, The effect of temperature upon the production of, by <i>Salmonella pullorum</i>	111
Importance, The, of feeds as a source of rosy milk organisms. Abstract...	37
Improved, An, method for the detection of hemolytic streptococcus carriers.....	219

Influence, The, of antigen-diluents on the intensity of flocculation in the precipitation test for syphilis. Abstract.....	47
— of surface tension upon the germination of bacterial spores.....	211
— of osmotic pressure upon spore germination.....	197
Isolation and study of an apparently widespread cellulose-fermenting anaerobe, <i>Cl. cellulosolvens</i> (N. sp.).....	167
Investigation, An, of the antiseptic and germicidal efficiency of Pyridium on various organisms. Abstract.....	54
Jamieson, W. A. and Powell, H. M. Merthiolate as a preservative for biological products. Abstract.....	55
Jennison, M. W. A bacteriological incubator for uniform temperatures with accurate control. Abstract.....	24
Jensen, Otto G., Lisse, Martin W. and Tittsler, Ralph P. A comparison of colorimetric and potentiometric methods for hydrogen ion determination of solid bacterial media, using a dilution method based on the buffer equation.....	383
Jordan, Elizabeth F. and Smith, George H. <i>Bacillus diphtheriae</i> in its relationship to bacteriophage.....	75
Kallas, J. G., Ruchhoft, C. C., Chinn, Ben, and Coulter, E. W. Coli-aerogenes differentiation in water analysis.....	407
Kendrick, Pearl and Hollon, Harriett C. Serologic and bacteriophagic relationships in a group of fecal streptococci. Abstract.....	49
Klimek, John, Hadley, Philip, Delves, Edna. The filtrable forms of bacteria. Abstract.....	7
Kopeloff, Nicholas and Upton, Margaret F. Serologic studies with <i>L. acidophilus</i> (Moro) and <i>L. bulgaricus</i> (Grigoroff). Abstract.....	51
Krumwiede, Charles, 1879-1930.....	141
Kulp, W. L., Conn, H. J., Chairman, Burke, Victor, Cohen, Barnett, Genung, Elizabeth F., Hall, Ivan, C. Report of committee on bacteriological technique: Progress during 1930. Abstract.....	1
Leifson, Einar. Bacterial spores.....	330
— Development of flagella on germinating spores.....	357
Leonard, Lewis T. <i>Bacillus radiobacter</i> in reference to commercial legume inoculants. Abstract.....	35
Lethal temperatures for <i>Br. abortus</i> with special reference to pasteurization. Abstract.....	54
Lisse, Martin W., Jensen, Otto G., and Tittsler, Ralph P. A comparison of colorimetric and potentiometric methods for hydrogen ion determination of solid bacterial media, using a dilution method based on the buffer equation.....	383
Lloyd, Blodwen. A marine denitrifying organism.....	89
Loeffler's blood serum, A modified, medium useful in the routine health department examination for diphtheria and streptococcus infections.....	119
Lupinus, Studies of the root-nodule organism of.....	273

Magoon, C. A. and Brunstetter, B. C. Studies upon bacterial spores. III. A contribution to the physiology of spore production in <i>B. mycoides</i> . Abstract.....	33
Maier, F. X. and Demeter, Karl J. Comparative investigations on the microbiological composition and the quality of butter. Abstract.....	41
Maneval, W. E. The staining of flagella of bacteria, with special reference to mordants.....	313
Marine, A, denitrifying organism.....	89
Massengale, O. H. and Prickett, Paul S. Ergosterol content of certain species of <i>Mycobacterium</i> and the effect of ergosterol (activated and unactivated) on their growth (final report). Abstract.....	9
McAlpine, J. G. and Plastring, Wayne N. Types of <i>Br. abortus</i> isolated from human, bovine, porcine and equine sources. Abstract.....	52
Medalia, Leon S., Bailey, Karl R. and Atwood, Catharine. A modified Loeffler's blood serum medium useful in the routine health department examination for diphtheria and streptococcus infections.....	119
Medin, The effect of sterilization of, upon their growth promoting properties toward bacteria.....	299
Merrill, Malcolm H. Studies on carbon metabolism of organisms of the genus <i>Mycobacterium</i> . II. Utilization of organic compounds in a synthetic medium.....	361
— Studies on carbon metabolism of organisms of the genus <i>Mycobacterium</i> . III. End products of carbohydrate utilization as determined in synthetic media cultures.....	375
Merthiolate as a preservative for biological products. Abstract.....	55
Method, A, for the determination of ethyl and butyl alcohols in fermentation mixtures. Abstract.....	20
Methods for determining the persistence of certain important bacteria in Swiss cheese. Abstract.....	40
Milas, Nicholas A. and Proctor, Bernard E. The bactericidal efficiency of certain organic peroxides. Abstract.....	55
Milk organisms, influence of associated cultures of. Studies on the influence of bacteria on the oxidation-reduction potential of milk. II.....	253
—, Influence of pure cultures of. Studies on the influence of bacteria on the oxidation-reduction potential of milk. I.....	239
— serum, Action of proteolytic bacteria on. Studies on the proteolytic bacteria of milk. V.....	263
—, Studies on the influence of bacteria on the oxidation-reduction potential of. I. Influence of pure cultures of milk organisms.....	239
—, ————. II. Influence of associated cultures of milk organisms.....	253
—, Studies on the proteolytic bacteria of. V. Action of proteolytic bacteria on milk serum.....	263
Modified, A, Loeffler's blood serum medium useful in the routine health department examination for diphtheria and streptococcus infections.....	119
Moore, Marjorie B. and Cromwell, H. W. Studies on pollen and pollen extracts. VI. Rabbit skin reactions in quantitative estimation of activity of pollen extracts. Abstract.....	59

Pierce, H. B. The effect of yeast on ammonia and indol production by bacteria in culture and in feces suspension.....	225
Plastring, Wayne N. and McAlpine, J. G. Types of <i>Br. abortus</i> isolated from human, bovine, porcine and equine sources. Abstract.....	52
Poe, Charles F. and Field, J. Thomas. The fermentation of polyhydric alcohols by the colon-aerogenes groups of bacteria. Abstract.....	11
Powell, H. M. and Jamieson, W. A. Merthiolate as a preservative for biological products. Abstract.....	55
—, Waldo, John H. and Shonle, H. A. The bacteriological evaluation of some new water soluble organomercury compounds.....	323
Prescott, Samuel C. and Bates, P. K. On the relation of refrigeration temperatures to the rate of growth of certain specific types of bacteria causing food spoilage. Abstract.....	25
—, and —. The reduction of the number of organisms in water as a result of freezing in domestic refrigerators. Abstract.....	26
—, — and Needle, H. C. The effect of discontinuous refrigeration on bacteria in foods. Abstract.....	25
—, Hale, J. R., and White, G. E. Observations on the bacteriology of slimy beef. Abstract.....	28
Presence of fusiform bacilli and spirochetes in the mouths of a group of young adults. Abstract.....	44
Prickett, Paul S. Thermophilic microorganisms from imported canned shallots (onions). Abstract.....	29
— and Massengale, O. N. Ergosterol content of certain species of <i>Mycobacterium</i> and the effect of ergosterol (activated and unactivated) on their growth (final report). Abstract.....	9
Problem, The, of ultrafiltration. Abstract.....	7
Proctor, Bernard E. An investigation of the antiseptic and germicidal efficiency of Pyridium on various organisms. Abstract.....	54
—, and Milas, Nicholas A. The bactericidal efficiency of certain organic peroxides. Abstract.....	55
Proteolytic bacteria of milk, Studies on the. V. Action of proteolytic bacteria on milk serum.....	263
Quantitative, A, study of the respiration of staphylococcus cultures lysed by bacteriophage.....	143
Rabbit skin reactions in quantitative estimation of activity of pollen extracts. Studies on pollen and pollen extracts. VI. Abstract.....	59
Reciprocal effects of the relationship of bacteriology and medicine.....	61
Reduction, The, of the number of organisms in water as a result of freezing in domestic refrigerators. Abstract.....	26
Reed, Guilford B. Dissociation of <i>Myc. leprae</i> . Abstract.....	12
Relation, On the, of refrigeration temperatures to the rate of growth of certain specific types of bacteria causing food spoilage. Abstract.....	25
Report of committee on bacteriological technic: Progress during 1930. Abstract.....	1

Rettger, L. F. and Casman, E. P. Some factors limiting bacterial growth at higher temperatures. Abstract.....	27
---- and Cowles, Philip B. Isolation and study of an apparently widespread cellulose-fermenting anaerobe, <i>Cl. cellulolyticus</i> (N. sp.?).....	167
---- and Weinstein, Louis. Biological and chemical studies of the lactobacillus genus with special reference to xylose fermentation. Abstract...	4
Robertson, A. H. and Schacht, F. L. Observed variations in bacterial counts on the same plate. Abstract.....	22
Robinson, G. H. and Taylor, F. A. The effect of urine on hemolytic streptococci. Abstract.....	49
Rogers, L. A. and Whittier, E. O. The continuous lactic fermentation of whey. Abstract.....	37
Root nodule organisms of <i>Lupinus</i> , Studies of the.....	273
Ruchhoft, C. C., Kallas, J. G., Chinn, Ben, and Coulter, E. W. Coliaerogenes differentiation in water analysis.....	407
Rupp, Philip, and Frazier, William C. Studies on the proteolytic bacteria of milk. V. Action of proteolytic bacteria on milk serum.....	263
<i>Salmonella pullorum</i> , The effect of temperature upon the production of hydrogen sulphide by.....	111
Saprophytic, Do, and secondary bacteria occurring in the respiratory tracts of domestic fowl in infectious trachitis and in health produce agglutinins in the blood? Abstract.....	45
---- and secondary microorganisms occurring in the respiratory tracts of domestic fowls and chickens in health and disease.....	97
Schacht, F. L. and Robertson, A. H. Observed variations in bacterial counts on the same plate. Abstract.....	23
Serologic and bacteriophage relationships in a group of fecal streptococci. Abstract.....	49
---- studies with <i>L. acidophilus</i> (Moro) and <i>L. bulgaricus</i> (Grigoroff). Abstract.....	51
Shenle, H. A., Waldo, John H. and Powell, H. M. The bacteriological evaluation of some new water soluble organomercury compounds.....	323
Shwartzman, Gregory. The phenomenon of local skin reactivity to various bacterial culture filtrates. A review. Abstract.....	56
Smith, George H. and Jordan, Elizabeth F. <i>Bacillus diphtheriae</i> in its relationship to bacteriophage.....	75
Spore germination, Influence of osmotic pressure upon.....	197
Spray, Robb Spalding. Demonstration of a simple anaerobic culture dish. Abstract.....	23
Squibb, E. R. and Sons. A comparison of methods for determination of potency as observed on a group of horses immunized against diphtheria. Abstract.....	46
Staining, The, of flagella of bacteria, with special reference to mordants.....	313
Staphylococcus cultures lysed by bacteriophage. A quantitative study of the respiration of.....	143
----, Dissociation in an encapsulated.....	157

- Stark, C. N. and Foter, M. J. The importance of feeds as a source of ropy milk organisms. Abstract..... 37
- and Herrington, B. L. The drying of bacteria and the viability of dry bacterial cells. Abstract..... 13
- Stearn, A. E. and Stearn, E. W. The characteristics of certain microorganisms as modified by the reaction of the medium. Abstract..... 18
- Steenken, William, Jr. and Petroff, S. A. Biological studies of the acid-fast organism. Abstract..... 11
- Sterilization of media, The effect of, upon their growth promoting properties toward bacteria..... 299
- Streptococcus infections, A modified Loeffler's blood serum medium useful in the routine health department examination for diphtheria and..... 119
- Studies of the properties of a bouillon filtrate of the gonococcus. Abstract.. 58
- of the root-nodule organism of Lupinus..... 273
- Studies of tularemia infection in rabbits and guinea pigs. Abstract..... 50
- on carbon metabolism of organisms of the genus *Mycobacterium*. II. Utilization of organic compounds in a synthetic medium..... 361
- — — — —, III. End products of carbohydrate utilization as determined in synthetic media cultures..... 375
- on pollen and pollen extracts. VI. Rabbit skin reactions in quantitative estimation of activity of pollen extracts. Abstract..... 59
- on the eye-forming organism of Swiss cheese. Abstract..... 39
- on the influence of bacteria on the oxidation-reduction potential of milk. I. Influence of pure cultures of milk organisms..... 239
- — — — —, II. Influence of associated cultures of milk organisms..... 253
- on the precipitin reaction. III. Further studies on conditions influencing the formation of precipitates. Abstract..... 47
- on the proteolytic bacteria of milk. V. Action of proteolytic bacteria on milk serum..... 263
- on typhus fever. Abstract..... 43
- upon bacterial spores. III. A contribution to the physiology of spore production in *B. mycoides*. Abstract..... 33
- Study, A, of the action of short electric waves on several biological substances with special reference to diphtheria toxin. Abstract..... 59
- Surface tension, influence of, upon the germination of bacterial spores.... 211
- Synthetic media cultures, End products of carbohydrate utilization as determined in. Studies on carbon metabolism of organisms of the genus *Mycobacterium*. III..... 375
- medium, Utilization of organic compounds in a. Studies of carbon metabolism of organisms of the genus *Mycobacterium*. II..... 361
- Szymonowski, Wacław T. and Hicks, Robert Alan. A study of the action of short electric waves on several biological substances with special reference to diphtheria toxin. Abstract..... 59
- Tanner, F. W. and Burrows, William. The viability of yeasts in sucrose solutions. Abstract..... 32
- and Wallace, G. I. The effect of concentrated salt and sugar solutions on the thermo-death times of molds. Abstract..... 32

- Taylor, F. A. and Robinson, G. H. The effect of urine on hemolytic streptococci. Abstract..... 49
- Temperature, The effect of, upon the production of hydrogen sulphide by *Salmonella pullorum*..... 111
- Thermophilic microorganisms from imported canned shalots (onions). Abstract..... 29
- Thompson, Luther. The occurrence of diphtheroids in routine blood cultures. Abstract..... 45
- Time-temperature-humidity relations in dried fruit pasteurization. Abstract..... 30
- Tittsler, Ralph P. The effect of temperature upon the production of hydrogen sulphide by *Salmonella pullorum*..... 111
- , Lisse, Martin W., and Jensen, Otto G. A comparison of colorimetric and potentiometric methods for hydrogen ion determination of solid bacterial media, using a dilution method based on the buffer equation.... 383
- Types of *Br. abortus* isolated from human, bovine, porcine and equine sources. Abstract..... 52
- Upton, Margaret F. and Kopeloff, Nicholas. Serologic studies with *L. acidophilus* (Moro) and *L. bulgaricus* (Grigoroff). Abstract..... 51
- Van Suchtelen, F. Hesselink, Humus as a source of energy for soil microorganisms, and the energy liberated by their activity. Abstract..... 33
- Van Vranken, Marjorie and Hall, Ivan C. A comparative and taxonomic study of thirty-eight strains of *Bacillus tertius* (Henry). Abstract.... 2
- Viability, The, of yeasts in sucrose solutions. Abstract..... 32
- Waldo, John H., Shonle, H. A. and Powell, H. M. The bacteriological evaluation of some new water soluble organomercury compounds..... 323
- Wallace, G. I. and Tanner, F. W. The effect of concentrated salt and sugar solutions on the thermo-death times of molds. Abstract..... 32
- Water analysis, *Coli-aerogenes* differentiation in..... 407
- soluble organomercury compounds, The bacteriological evaluation of some new..... 323
- Weaver, R. H. Electrophoresis studies on *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* cultures. Abstract..... 51
- Weinstein, Louis and Rettger, Leo F. Biological and chemical studies of the lactobacillus genus with special reference to xylose fermentation. Abstract.... 4
- Weiss, Emil. A direct method for the determination of the H-ion concentration of cultures. Abstract..... 22
- . The influence of antigen-diluents on the intensity of flocculation in the precipitation test for syphilis. Abstract..... 47
- Werkman, C. H., Fulmer, Ellis I., and Williams, Arthur L. The effect of sterilization of media upon their growth promoting properties toward bacteria..... 299
- and Gillen, G. Bacteria producing trimethylene glycol. Abstract.... 6
- and Osburn, O. L. A method for the determination of ethyl and butyl alcohols in fermentation mixtures. Abstract..... 20

White, G. E., Prescott, Samuel C., and Hale, J. F. Observations on the bacteriology of slimy beef. Abstract.....	26
White, Harry, Huntoon, F. M. and Funk, E. H. Biochemical studies of bacterial derivatives. XV. Skin reactions in man: A comparison of tuberculin O.T., human tubercle bacillus protein MA 100 and timothy bacillus protein MA 100. Abstract.....	57
Whitman, Loring and Mueller, J. Howard. An improved method for the detection of hemolytic streptococcus carriers.....	219
Whittier, Earle O. and Frazier, William C. Studies on the influence of bacteria on the oxidation-reduction potential of milk. I. Influence of associated cultures of milk organisms.....	239
— and —. —————, — II. Influence of associated cultures of milk organisms.....	253
— and Rogers, L. A. The continuous lactic fermentation of whey. Abstract.....	37
Williams, Arthur L., Fulmer, Ellis I. and Werkman, C. H. The effect of sterilization of media upon their growth promoting properties toward bacteria.....	299
Williams, O. B. The effects of alkaloids on bacteria. Abstract.....	55
Wing, Helen U. and Frazier, William C. Studies on the eye-forming organism of Swiss cheese. Abstract.....	39
Yeast, The effect of, on ammonia and indol production by bacteria in culture and in feces suspension.....	225
Zinsser, Hans and Castaneda, M. Ruiz. Studies on typhus fever. Abstract..	43

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